

SYNTHETIC MEMBRANE ANCHORS

FIELD OF INVENTION

5 The invention relates to synthetic molecules that spontaneously and stably incorporate into lipid bi-layers, including cell membranes. Particularly, although not exclusively, the invention relates to the use of these molecules as synthetic membrane anchors or synthetic molecule constructs to effect qualitative and quantitative changes in the expression of cell surface antigens.

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BACKGROUND

Cell surface antigens mediate a range of interactions between cells and their environment. These interactions include cell-cell interactions, cell-surface interactions and cell-solute

15 interactions. Cell surface antigens also mediate intra-cellular signalling.

Cells are characterised by qualitative and quantitative differences in the cell surface antigens expressed. Qualitative and quantitative changes in the cell surface antigens expressed alter both cell function (mode of action) and cell functionality (action served).

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Being able to effect qualitative and/or quantitative changes in the surface antigens expressed by a cell has diagnostic and therapeutic value. Transgenic and non-transgenic methods of effecting qualitative and/or quantitative changes in the surface antigens expressed by a cell are known.

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Protein painting is a non-transgenic method for effecting qualitative and/or quantitative changes in the surface antigens expressed by a cell. The method exploits the ability of GPI linked proteins to spontaneously anchor to the cell membrane via their lipid tails. The method described in the specification accompanying international application no. PCT/US98/15124 (WO 99/05255) includes the step of inserting a GPI linked protein isolated from a biological source into a membrane. Isolated GPI-anchored proteins are stated as having an unusual capacity to re-integrate with a cell-surface membrane.

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Cells exist in an aqueous environment. The cell membrane is a lipid bilayer that serves as a semi-permeable barrier between the cytoplasm of the cell and this aqueous environment. Localising antigens to the cell surface may also be achieved by the use of glycolipids as membrane anchors.

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The method described in the specification accompanying international application no. PCT/NZ02/00214 (WO 03/034074) includes the step of inserting a controlled amount of glycolipid into a membrane. The amount of glycolipid inserted is controlled to provide cells

with a desired level of antigen expression.

The method described in the specification accompanying international application no. PCT/NZ03/00059 (WO 03/087346) includes the step of inserting a modified glycolipid into a membrane as a "membrane anchor". The modified glycolipid provides for the localisation of antigens to the surface of the cell or multicellular structure. New characteristics may thereby be imparted on the cell or multicellular structure.

These methods typically include the isolation of a glycolipid or glycolipid-linked antigen from a biological source. The isolation of glycolipids or glycolipid-linked antigens from biological sources is costly, variable and isolatable amounts are often limited. Obtaining reagents from zoological sources for therapeutic use is particularly problematic, especially where the reagent or its derivative products are to be administered to a human subject.

Synthetic molecules for which the risk of contamination with zoo-pathogenic agents can be excluded are preferred. Synthetic counterparts for naturally occurring glycolipids and synthetic neo-glycolipids have been reported. However, for a synthetic glycolipid to be of use as a membrane anchor it must be able to spontaneously and stably incorporate into a lipid bi-layer from an aqueous environment. The utility of synthetic glycolipids in diagnostic or therapeutic applications is further limited to those synthetic glycolipids that will form a solution in saline.

Organic solvents and/or detergents used to facilitate the solubilization of glycolipids in saline must be biocompatible. Solvents and detergents must often be excluded or quickly removed as they can be damaging to some cell membranes. The removal of solvents or detergents from such preparations can be problematic.

Damage to cell membranes is to be avoided especially where the supply of cells or multicellular structures is limited, e.g. embryos, or the cells are particularly sensitive to perturbation, e.g. hepatocytes.

There exists a need for water soluble synthetic molecules that are functionally equivalent to naturally occurring glycolipids and glycolipid-linked antigens in respect of their ability to spontaneously and stably incorporate into lipid bi-layers, including cell membranes.

Providing such synthetic molecules would obviate the limitations of glycolipids and glycolipid-linked antigens isolated from biological sources and facilitate being able to effect qualitative and/or quantitative changes in the surface antigens expressed by a cell.

It is an object of this invention to provide such synthetic molecules and a method for their preparation. It is a further object of this invention to provide synthetic molecules for use in diagnostic and therapeutic applications. The preceding objects are to be read disjunctively

with the object to at least provide the public with a useful choice.

STATEMENTS OF INVENTION

5 In a **first** aspect the invention consists in a molecule of the structure R-S₂-L for use as a synthetic membrane anchor or in the preparation of synthetic molecule constructs where:

R is a chemically reactive functional group;

S₂ is a spacer linking R to L; and

10 L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids, and sphingosine derived diacyl- and dialkyl-lipids, including ceramide.

Preferably R is selected from the group including: *bis*(N-hydroxysuccinimidyl), *bis*(4-

15 *nitrophenyl*), *bis*(pentafluorophenyl), *bis*(pentachlorophenyl).

Preferably S₂ is selected from the group including: -CO(CH₂)₃CO-, -CO(CH₂)₄CO- (adipate (Ad)), and -CO(CH₂)₅CO-.

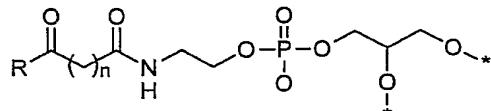
20 Preferably R and S₂ are ester linked.

Preferably L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids. More preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine,

25 phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably the lipid is derived from one or 30 more *cis*-destaurated fatty acids. Most preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

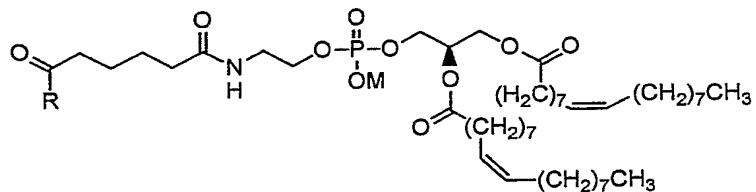
Preferably L is a glycerophospholipid and the molecule includes the substructure:

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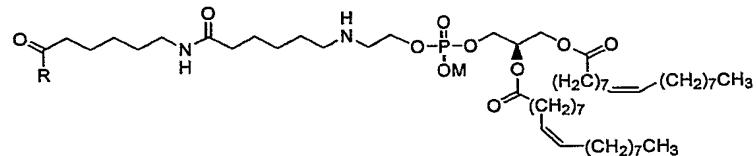


where n = 3 to 5 and * is other than H. Preferably n is 3.

In specific embodiments the molecule has the structure:

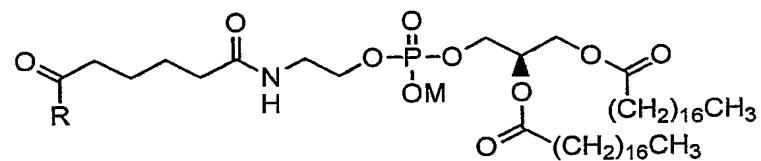


5 designated Ad-DOPE; the structure:



designated sp₁-Ad-DOPE; or the structure:

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designated Ad-DSPE.

15 M is typically H, but may be replaced by another monovalent cation such as Na⁺, K⁺ or NH₄⁺.

In a second aspect the invention consists in a synthetic molecule construct of the structure F-S₁-S₂-L where:

20 F is an antigen selected from the group consisting of carbohydrates, proteins, lipids,

lectins, avidins and biotin;

S₁-S₂ is a spacer linking F to L; and

L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids, and sphingosine derived diacyl- and dialkyl-lipids,

25 including ceramide.

Preferably the molecule is water soluble.

30 Preferably the molecule spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer. More preferably the molecule stably incorporates into the lipid bilayer.

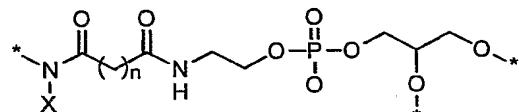
Preferably F, S₁, S₂ and L are covalently linked.

Preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes, antibodies (immunoglobulins), lectins, avidins, and biotin. Most preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes or antibodies (immunoglobulins).

Preferably L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids. More preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *cis*-11-trans-9-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably the lipid is derived from one or more *cis*-destaturated fatty acids. Most preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

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Preferably L is a glycerophospholipid and the molecule includes the substructure:



25 where n = 3 to 5, X is H or C, and * is other than H. Preferably n is 3.

S₁-S₂ is selected to provide a water soluble synthetic molecule construct.

In a first embodiment F is a naturally occurring or synthetic glycopote. Preferably F is a naturally occurring or synthetic glycopote consisting of three (trisaccharide) or more sugar units. More preferably F is a glycopote selected from the group consisting of lacto-neo-tetraosyl, lactotetraosyl, lacto-nor-hexaosyl, lacto-iso-octaosyl, globotetraosyl, globo-neo-tetraosyl, globopentaosyl, gangliotetraosyl, gangliotriaosyl, gangliopentaosyl, isoglobotriaosyl, isoglobotetraosyl, mucotriaosyl and mucotetraosyl series of oligosaccharides. Most preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAc α 1-3(Fuca1-2)Gal β ; Gal α 1-3Gal β ; Gal β ; Gal α 1-3(Fuca1-2)Gal β ; NeuAco2-3Gal β ; NeuAco2-6Gal β ; Fuca1-2Gal β ; Gal β 1-4GlcNAc β 1-6(Gal β 1-4GlcNAc β 1-3)Gal β ; Fuca1-2Gal β 1-4GlcNAc β 1-6(Fuca1-2Gal β 1-4GlcNAc β 1-3)Gal β ; Fuca1-2Gal β 1-4GlcNAc β 1-6(NeuAco2-3Gal β 1-4GlcNAc β 1-3)Gal β ; NeuAco2-3Gal β 1-4GlcNAc β 1-6(NeuAco2-3Gal β 1-4GlcNAc β 1-3)Gal β ; Gal α 1-4Gal β 1-

4Glc; GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; GalNAc α 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; or GalNAc β 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc.

When F is a glycopote, L is a glycerophospholipid and S₂ is selected from the group including:

5 -CO(CH₂)₃CO-, -CO(CH₂)₄CO- (adipate), -CO(CH₂)₅CO- and -CO(CH₂)₅NHCO(CH₂)₅CO-, preferably S₁ is a C₃₋₅-aminoalkyl selected from the group consisting of: 3-aminopropyl, 4-aminobutyl, or 5-aminopentyl. More preferably S₁ is 3-aminopropyl.

In a second embodiment F is a molecule that mediates a cell-cell or cell-surface interaction.

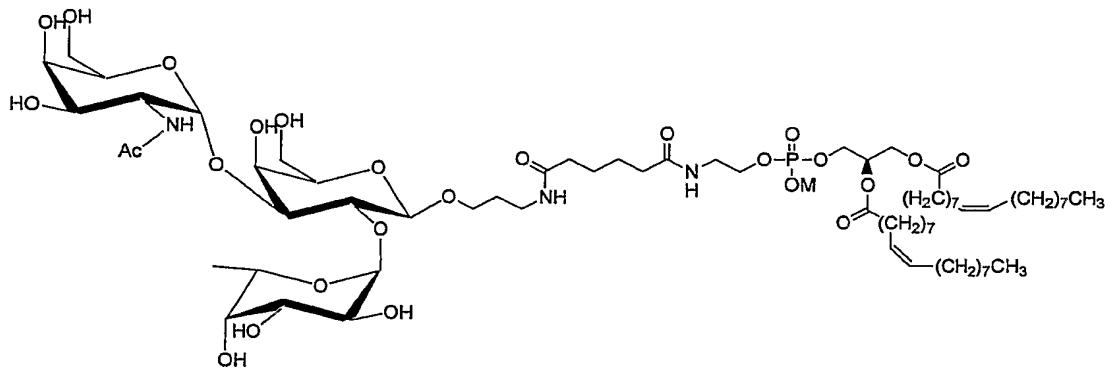
10 Preferably F is carbohydrate, protein or lipid with an affinity for a component expressed on a targeted cell or surface. More preferably F has an affinity for a component expressed on epithelial cells or extra-cellular matrices. Yet more preferably F has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium. Most preferably the component expressed on the epithelial cells or the extra-cellular matrix of the endometrium can be a naturally expressed component or an exogenously incorporated component.

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In a third embodiment F is a molecule that mediates a cell-solute interaction. Preferably F is a ligand for a binding molecule where the presence of the binding molecule is diagnostic for a pathological condition. More preferably F is a ligand for an antibody (immunoglobulin).

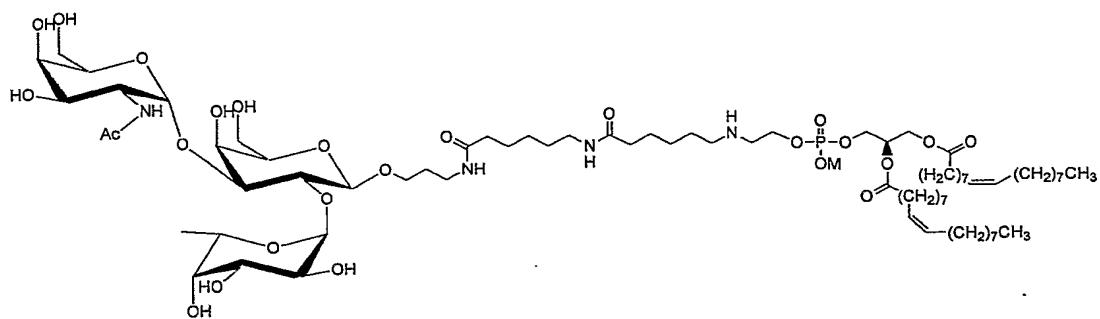
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In specific embodiments the water soluble synthetic molecule construct has the structure:

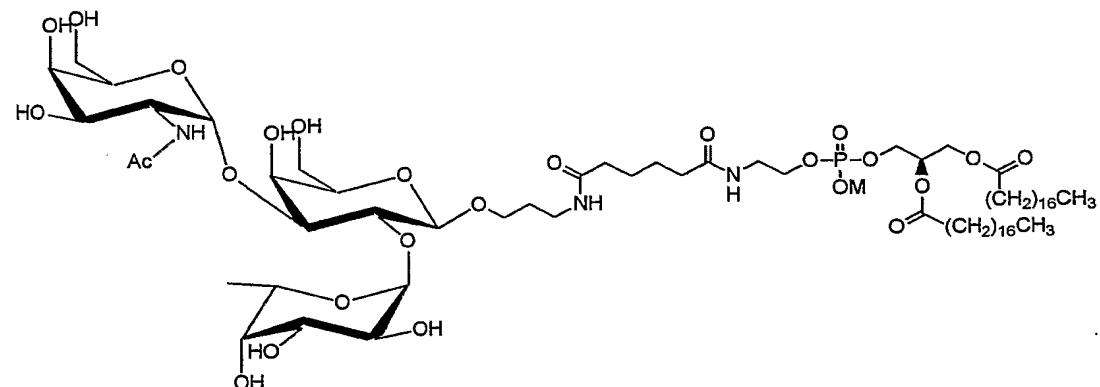


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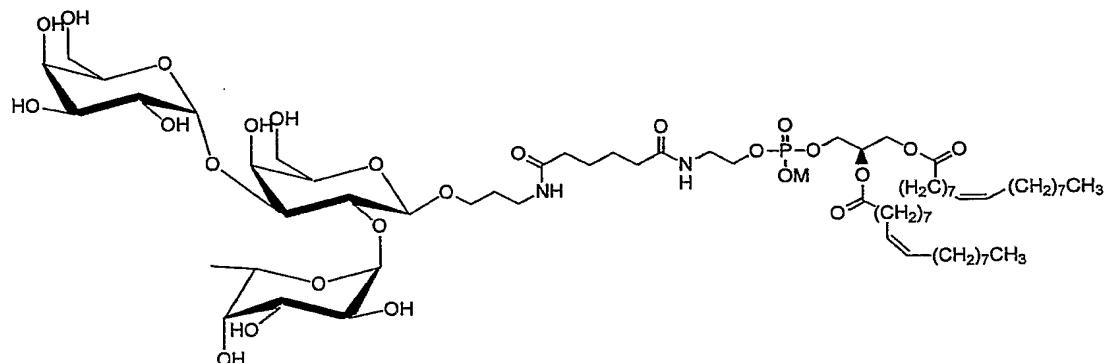
designated A_{tri}-sp-Ad-DOPE (I); the structure:

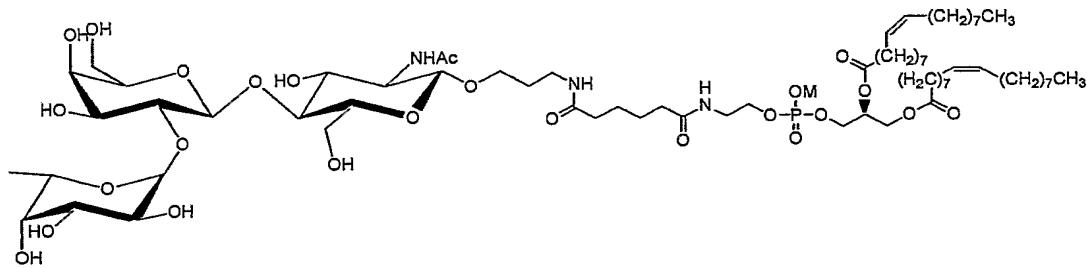
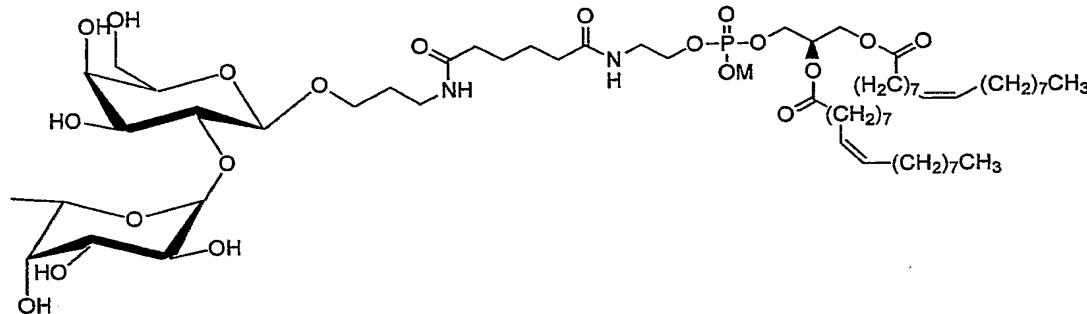
designated A_{tri} -sp-sp₁-Ad-DOPE (**II**); the structure:

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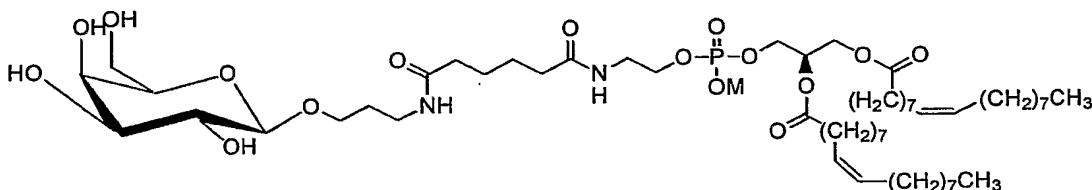
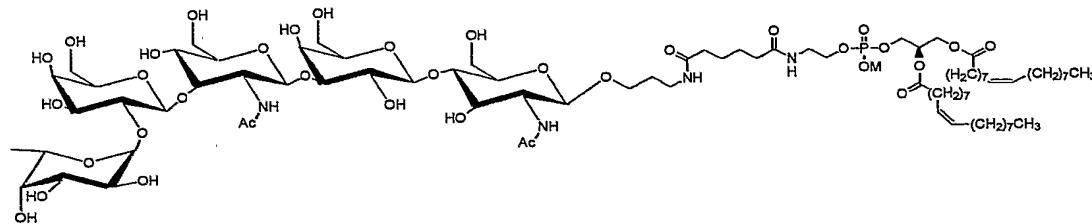
designated A_{tri} -sp-Ad-DSPE (**III**); the structure

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designated B_{tri} -sp-Ad-DOPE (**VI**); the structure:

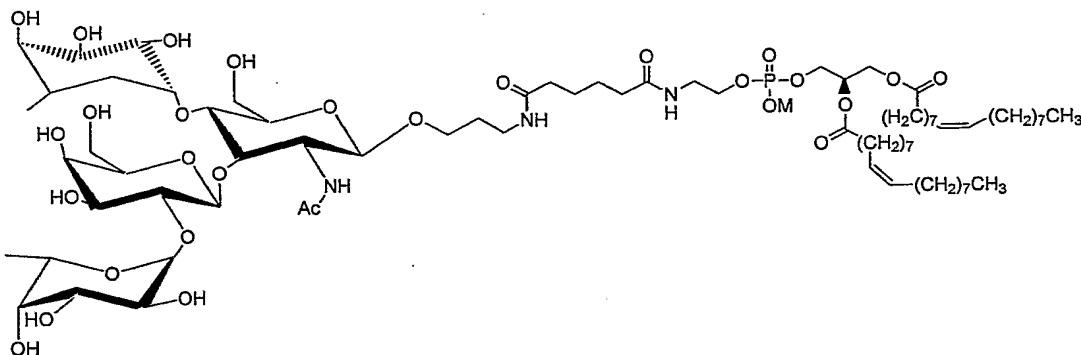
designated H_{tri}-sp-Ad-DOPE (VII); the structure:

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designated H_{di}-sp-Ad-DOPE (VIII); the structure:10 designated Galβ₁-sp-Ad-DOPE (IX); the structure:

designated Fuca1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc-sp-Ad-DOPE (XII); or the structure:

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designated Fuca1-2Galβ1-3(Fuca1-4)GlcNAc-sp-Ad-DOPE (XIII).

5 M is typically H, but may be replaced by another monovalent cation such as Na⁺, K⁺ or NH₄⁺.

In a third aspect the invention consists in a method of preparing a synthetic molecule construct of the structure F-S₁-S₂-L including the steps:

10 1. Reacting an activator (A) with a lipid (L) to provide an activated lipid (A-L);
 2. Derivatising an antigen (F) to provide a derivatised antigen (F-S₁); and
 3. Condensing A-L with F-S₁ to provide the molecule;

where:

15 A is an activator selected from the group including: bis(N-hydroxysuccinimidyl), bis(4-nitrophenyl), bis(pentafluorophenyl), bis(pentachlorophenyl) esters of carbodioic acids (C₃ to C₇);
 L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids, and sphingosine derived diacyl- and dialkyl-lipids, including ceramide.
 20 F is an antigen selected from the group consisting of carbohydrates, proteins, lipids, lectins, avidins and biotin; and
 S₁-S₂ is a spacer linking F to L where S₁ is selected from the group including: primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine; and S₂ is absent or selected from the group including: -CO(CH₂)₃CO-, -CO(CH₂)₄CO- (adipate), and -CO(CH₂)₅CO-.

25 Preferably the molecule is water soluble.

30 Preferably the molecule spontaneously incorporates into a lipid bi-layer when a solution of the molecule is contacted with the lipid bi-layer. More preferably the molecule stably incorporates into the lipid bilayer.

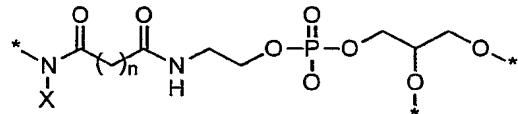
Preferably F, S₁, S₂ and L are covalently linked.

Preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes, antibodies (immunoglobulins), lectins, avidins, and biotin. Most preferably F is selected from the group consisting of naturally occurring or synthetic glycotopes or antibodies (immunoglobulins).

Preferably L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids. More preferably L is selected from the group consisting of: diacylglycerolipids, phosphatidate, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, and diphosphatidyl glycerol derived from one or more of *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid. More preferably the lipid is derived from one or more *cis*-destaurated fatty acids. Most preferably L is selected from the group consisting of: 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-O-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) and *rac*-1,2-dioleoylglycerol (DOG).

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Preferably L is a glycerophospholipid and the molecule includes the substructure:



25 where n = 3 to 5, X is H or C, and * is other than H. Preferably n is 3.

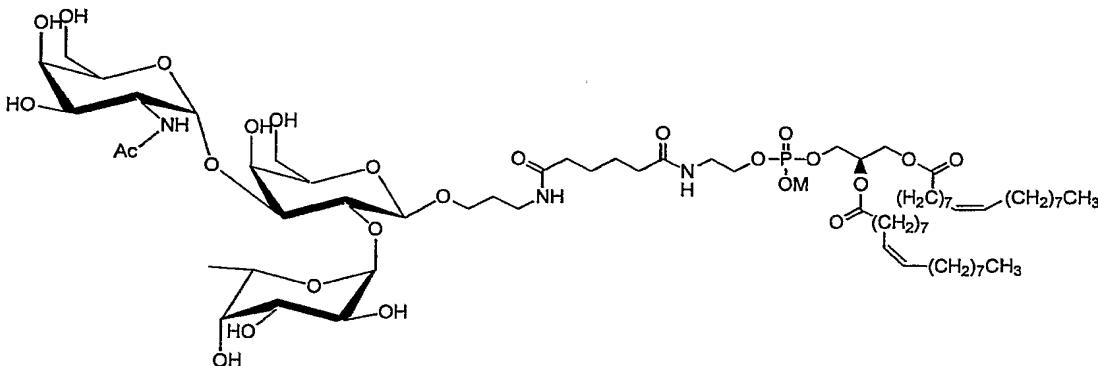
Preferably A (R-S₂) and S₁ are selected to provide a water soluble synthetic molecule construct.

30 In a first embodiment F is a naturally occurring or synthetic glycopote. Preferably F is a naturally occurring or synthetic glycopote consisting of three (trisaccharide) or more sugar units. More preferably F is a glycopote selected from the group consisting of lacto-neo-tetraosyl, lactotetraosyl, lacto-nor-hexaosyl, lacto-iso-octaosyl, globotetraosyl, globo-neo-tetraosyl, globopentaosyl, gangliotetraosyl, gangliotriaosyl, gangliopentaosyl, isoglobotriaosyl, isoglobotetraosyl, mucotriaosyl and mucotetraosyl series of oligosaccharides. Most preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAc1-3(Fuca1-2)Galβ; Galα1-3Galβ; Galβ; Galα1-3(Fuca1-2)Galβ; NeuAca2-3Galβ; NeuAca2-6Galβ; Fuca1-2Galβ; Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ; Fuca1-2Galβ1-4GlcNAcβ1-6(Fuca1-2Galβ1-4GlcNAcβ1-3)Galβ; Fuca1-2Galβ1-4GlcNAcβ1-6(NeuAca2-3Galβ1-4GlcNAcβ1-

3)Gal β ; NeuAca2-3Gal β 1-4GlcNAc β 1-6(NeuAca2-3Gal β 1-4GlcNAc β 1-3)Gal β ; Gal α 1-4Gal β 1-4Glc; GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; GalNAc α 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; or GalNAc β 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc.

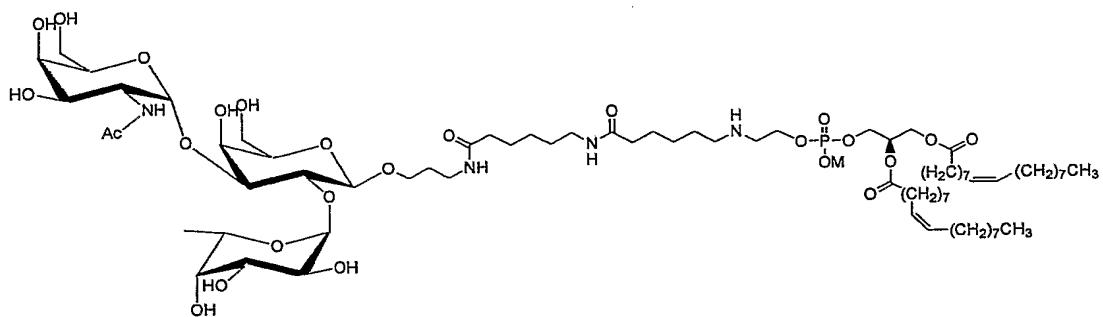
- 5 When F is a glycotope, L is a glycerophospholipid and S₂ is selected from the group including: -CO(CH₂)₃CO-, -CO(CH₂)₄CO- (adipate), -CO(CH₂)₅CO- (e.g. A is bis(N-hydroxysuccinimidyl) adipate), preferably S₁ is a C₃₋₅-aminoalkyl selected from the group consisting of: 3-aminopropyl, 4-aminobutyl, or 5-aminopentyl). More preferably S₁ is 3-aminopropyl.
- 10 In a second embodiment F is a molecule that mediates a cell-cell or cell-surface interaction. Preferably F is carbohydrate, protein or lipid with an affinity for a component expressed on a targeted cell or surface. More preferably F has an affinity for a component expressed on epithelial cells or extra-cellular matrices. Yet more preferably F has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium. Most
- 15 preferably the component expressed on the epithelial cells or the extra-cellular matrix of the endometrium can be a naturally expressed component or an exogenously incorporated component.
- In a third embodiment F is a molecule that mediates a cell-solute interaction. Preferably F is a
- 20 ligand for a binding molecule where the presence of the binding molecule is diagnostic for a pathological condition. More preferably F is a ligand for an antibody (immunoglobulin).

In specific embodiments the water soluble synthetic molecule construct has the structure:

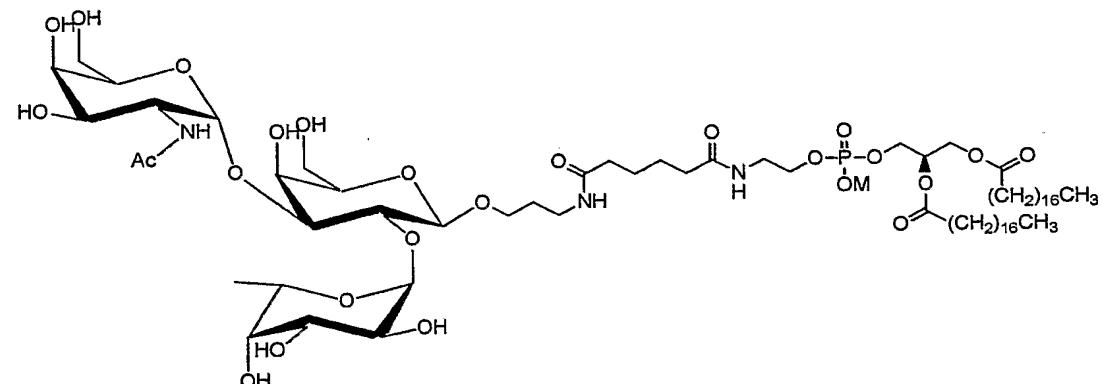


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designated A_{Hi}-sp-Ad-DOPE (I); the structure:

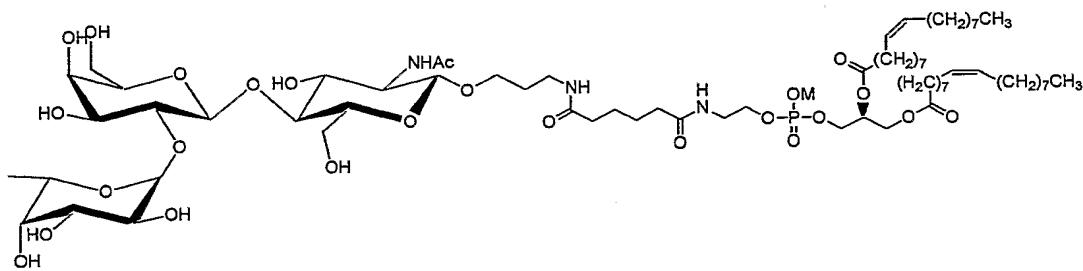
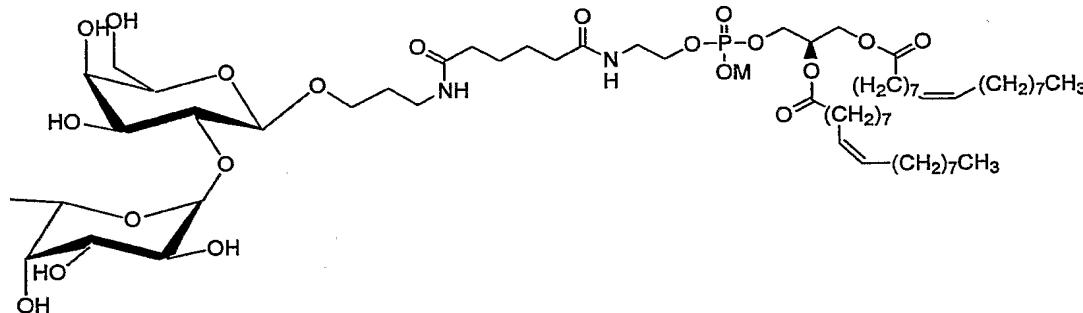
designated A_{tri} -sp-sp₁-Ad-DOPE (**II**); the structure:

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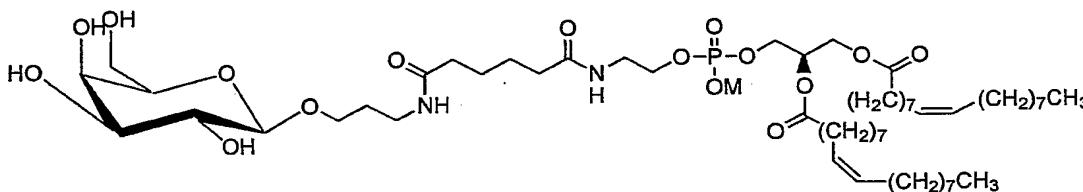
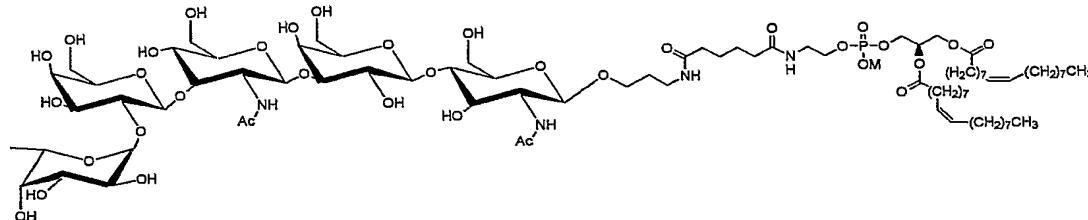
designated A_{tri} -sp-Ad-DSPE (**III**); the structure

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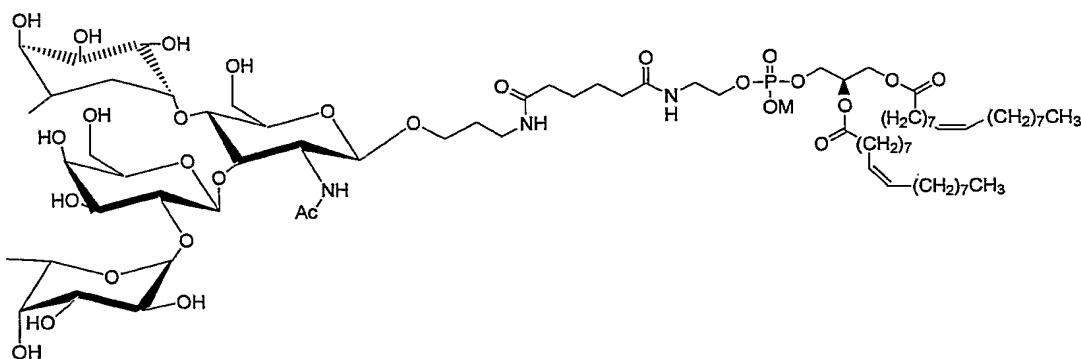
designated B_{tri} -sp-Ad-DOPE (**VI**); the structure:

designated H_{tri} -sp-Ad-DOPE (VII); the structure:

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designated H_{dr} -sp-Ad-DOPE (VIII); the structure:10 designated Gal β_1 -sp-Ad-DOPE (IX); the structure:designated Fuca1-2Gal β_1 -3GlcNAc β_1 -3Gal β_1 -4GlcNAc-sp-Ad-DOPE (XII); or the structure:

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designated Fu α 1-2Gal β 1-3(Fu α 1-4)GlcNAc-sp-Ad-DOPE (XIII).

5 M is typically H, but may be replaced by another monovalent cation such as Na⁺, K⁺ or NH₄⁺.

In a **fourth** aspect the invention consists in a water soluble synthetic molecule construct prepared by a method according to the third aspect of the invention.

10 In a **fifth** aspect the invention consists in a method of effecting qualitative and/or quantitative changes in the surface antigens expressed by a cell or multi-cellular structure including the step:

15 1. Contacting a suspension of the cell or multi-cellular structure with a synthetic molecule construct according to the second aspect or fourth aspect of the invention for a time and at a temperature sufficient to effect the qualitative and/or quantitative change in the surface antigens expressed by the cell or multi-cellular structure.

Preferably the cell or multi-cellular structure is of human or murine origin.

20 Preferably the concentration of the water soluble synthetic molecule construct in the suspension is in the range 0.1 to 10 mg/mL.

25 Preferably the temperature is in the range 2 to 37 °C. More preferably the temperature is in the range 2 to 25 °C. Most preferably the temperature is in the range 2 to 4 °C.

In a first embodiment the cell is a red blood cell.

30 In this embodiment preferably F is selected from the group of glycotopes comprising the terminal sugars GalNAc α 1-3(Fu α 1-2)Gal β ; Gal α 1-3Gal β ; Gal β ; Gal α 1-3(Fu α 1-2)Gal β ; NeuAco2-3Gal β ; NeuAco2-6Gal β ; Fu α 1-2Gal β ; Gal β 1-4GlcNAc β 1-6(Gal β 1-4GlcNAc β 1-3)Gal β ; Fu α 1-2Gal β 1-4GlcNAc β 1-6(Fu α 1-2Gal β 1-4GlcNAc β 1-3)Gal β ; Fu α 1-2Gal β 1-4GlcNAc β 1-6(NeuAco2-3Gal β 1-4GlcNAc β 1-3)Gal β ; NeuAco2-3Gal β 1-4GlcNAc β 1-

6(NeuAca2-3Gal β 1-4GlcNAc β 1-3)Gal β ; Gal α 1-4Gal β 1-4Glc; GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; GalNAc α 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc; or GalNAc β 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc. More preferably F is selected from the group of glycotopes consisting of the oligosaccharides GalNAc α 1-3(Fuca1-2)Gal β and Gal α 1-3(Fuca1-2)Gal β .

5

Preferably the synthetic molecule construct is selected from the group including: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp_{p1}-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**); Gal β _i-sp-Ad-DOPE (**IX**); Fuca1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β 1-3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**).

10

In a second embodiment the multi-cellular structure is an embryo.

15 In this embodiment preferably F is an attachment molecule where the attachment molecule has an affinity for a component expressed on the epithelial cells or the extra-cellular matrix of the endometrium.

20 The component expressed on the epithelial cells or the extra-cellular matrix of the endometrium can be a naturally expressed component or an exogenously incorporated component.

25 Preferably the synthetic molecule construct is selected from the group including: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp_{p1}-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**); Gal β _i-sp-Ad-DOPE (**IX**); Fuca1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β 1-3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**).

In a third embodiment the cell is red blood cell.

30 In this embodiment preferably F is a ligand for a binding molecule where the presence of the binding molecule is diagnostic for a pathological condition. More preferably F is a ligand for an antibody (immunoglobulin).

35 In a sixth aspect the invention consists in a cell or multi-cellular structure incorporating a water soluble synthetic molecule construct according to the second or fourth aspect of the invention.

Preferably the cell or multi-cellular structure is of human or murine origin.

40 In a first embodiment the cell is a red blood cell incorporating a water soluble synthetic molecule construct selected from the group including: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp_{p1}-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**);

Gal β_1 -sp-Ad-DOPE (**IX**); Fuca1-2Gal β_1 -3GlcNAc β_1 -3Gal β_1 -4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β_1 -3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**).

In a second embodiment the multi-cellular structure is an embryo incorporating a water soluble synthetic molecule construct selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp₁-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**); Gal β_1 -sp-Ad-DOPE (**IX**); Fuca1-2Gal β_1 -3GlcNAc β_1 -3Gal β_1 -4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β_1 -3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**).

10 In a **seventh** aspect the invention consists in a kit comprising a dried preparation or solution of a molecule according to the first aspect of the invention, or a dried preparation or solution of a water soluble synthetic molecule construct according to the second or fourth aspect of the invention.

15 Preferably the molecule according to the first aspect of the invention is selected from the group consisting of: Ad-DOPE; sp₁-Ad-DOPE; and Ad-DSPE.

Preferably water soluble synthetic molecule construct according to the second or fourth aspect of the invention is selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp₁-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**); Gal β_1 -sp-Ad-DOPE (**IX**); Fuca1-2Gal β_1 -3GlcNAc β_1 -3Gal β_1 -4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β_1 -3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**).

20 In an **eighth** aspect the invention consists in a kit comprising a suspension in a suspending solution of cells or multi-cellular structures according to the sixth aspect of the invention.

25 Preferably the suspending solution is substantially free of lipid.

30 Preferably the cell or multi-cellular structure is of human or murine origin.

35 Preferably the cells are red blood cells that do not naturally express A- or B-antigen and incorporate a water soluble synthetic molecule construct selected from the group consisting of: A_{tri}-sp-Ad-DOPE (**I**); A_{tri}-sp₁-Ad-DOPE (**II**); A_{tri}-sp-Ad-DSPE (**III**); B_{tri}-sp-Ad-DOPE (**VI**); H_{tri}-sp-Ad-DOPE (**VII**); H_{di}-sp-Ad-DOPE (**VIII**); Gal β_1 -sp-Ad-DOPE (**IX**); Fuca1-2Gal β_1 -3GlcNAc β_1 -3Gal β_1 -4GlcNAc-sp-Ad-DOPE (**XII**); and Fuca1-2Gal β_1 -3(Fuca1-4)GlcNAc-sp-Ad-DOPE (**XIII**). More preferably the cells are sensitivity controls.

40 In a **ninth** aspect the invention consists in a pharmaceutical preparation comprising a dried preparation or solution of a water soluble synthetic molecule construct according to the second or fifth aspect of the invention.

Preferably the pharmaceutical preparation is in a form for administration by inhalation.

Preferably the pharmaceutical preparation is in a form for administration by injection.

5 In an tenth aspect the invention consists in a pharmaceutical preparation comprising cells or multi-cellular structures according to the sixth aspect of the invention.

Preferably the cells or multi-cellular structures are of human or murine origin.

10 Preferably the pharmaceutical preparation is in a form for administration by inhalation.

Preferably the pharmaceutical preparation is in a form for administration by injection.

DETAILED DESCRIPTION

15 The synthetic molecule constructs of the invention spontaneously and stably incorporate into a lipid bi-layer, such as a membrane, when a solution of the molecule is contacted with the lipid bi-layer. Whilst not wishing to be bound by theory it is believed that the insertion into the membrane of the lipid tails of the lipid (L) is thermodynamically favoured. Subsequent

20 disassociation of the synthetic molecule construct from the lipid membrane is believed to be thermodynamically unfavoured. Surprisingly, the synthetic molecule constructs identified herein have also been found to be water soluble.

25 The synthetic molecule constructs of the invention are used to transform cells resulting in qualitative and/or quantitative changes in the surface antigens expressed. It will be recognised that the transformation of cells in accordance with the invention is distinguished from transformation of cells by genetic engineering. The invention provides for phenotypic transformation of cells without *genetic* transformation.

30 In the context of this description the term "transformation" in reference to cells is used to refer to the insertion or incorporation into the cell membrane of exogenously prepared synthetic molecule constructs thereby effecting qualitative and quantitative changes in the cell surface antigens expressed by the cell.

35 The synthetic molecule constructs of the invention comprise an antigen (F) linked to a lipid portion (or moiety) (L) via a spacer (S₁-S₂). The synthetic molecule constructs can be prepared by the condensation of a primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine derivative of the antigen with an activated lipid. Methods of preparing neoglycoconjugates have been reviewed (Bovin, N. Biochem. Soc. Symp., 69, 143-160).

40 A desired phenotypic transformation may be achieved using the synthetic molecule constructs

A desired phenotypic transformation may be achieved using the synthetic molecule constructs of the invention in a one step method or a two step method. In the one step method the water soluble synthetic molecule construct (F-S₁-S₂-L) comprises the surface antigen as F.

5 In the two step method the synthetic molecule construct (F-S₁-S₂-L) comprises an antigen (F) that serves as a functional group to which a surface antigen can be linked following insertion of the synthetic molecule construct into the membrane. The functional group can be a group such as a lectin, avidin or biotin. When used in the two step method the synthetic molecule construct is acting as a synthetic membrane anchor.

10 In accordance with the invention the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine and the activator of the lipid are selected to provide a synthetic molecule construct that is water soluble and will spontaneously and stably incorporate into a lipid bi-layer when a solution of the synthetic molecule construct is contacted with the lipid bi-layer.

15 In the context of this description the phrase "water soluble" means a stable, single phase system is formed when the synthetic molecule construct is contacted with water or saline (such as PBS) in the absence of organic solvents or detergents, and the term "solution" has a corresponding meaning.

20 In the context of this description the phrase "stably incorporate" means that the synthetic molecule constructs incorporate into the lipid bi-layer or membrane with minimal subsequent exchange between the lipid bi-layer or membrane and the external aqueous environment of the lipid bi-layer or membrane.

The selection of the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine and the activator depends on the physico-chemical properties of the antigen (F) to be linked to the lipid (L).

25 It will be understood by those skilled in the art that for a non-specific interaction, such as the interaction between a diacyl- or dialkyl-glycerolipid and a membrane, structural and stereoisomers of naturally occurring lipids can be functionally equivalent. For example, it is contemplated by the inventors that diacylglycerol 2-phosphate could be substituted for phosphatidate (diacylglycerol 3-phosphate). Furthermore it is contemplated by the inventors that the absolute configuration of phosphatidate can be either R or S.

30 The inventors have determined that to prepare synthetic molecule constructs of the invention where the antigen (F) is an oligosaccharide selected from the group of glycotopes for A-, B- and H-antigens of the ABO blood groups, the primary aminoalkyl, secondary aliphatic aminoalkyl or primary aromatic amine, and the activator should be selected to provide a

Alternative structures of S ₁ -S ₂ for a water soluble synthetic molecule construct (F-S ₁ -S ₂ -L) where F is a carbohydrate (or other antigen) with similar physico-chemical properties to the carbohydrate portion of the A-, B- or H-antigens of the ABO blood groups and L is a glycerophospholipid (n, m independently = 2 to 5)	
S ₁ is selected from: -O(CH ₂) _n NH-	S ₂ is selected from: -CO(CH ₂) _n CO- or -CO(CH ₂) _m NHCO(CH ₂) _n CO-

It will be understood by one skilled in the art that once the structure of the spacer (S₁-S₂) has been determined for a given class of antigens, the same structure of the spacer can be

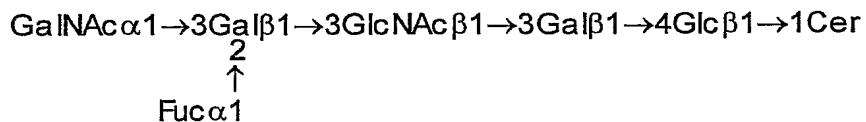
- 5 adopted to prepare synthetic molecule constructs of other classes of antigen with similar physico-chemical properties.
- For example, the structure of the spacer for synthetic molecule constructs (F-S₁-S₂-L) where F is a glycotope of the A-, B- and H-antigens of the ABO blood groups, may be the structure of
- 10 the spacer selected to prepare synthetic molecule constructs of other antigens with physico-chemical properties similar to the glycotopes of the A-, B- and H-antigens of the ABO blood groups.
- In principle the glycotope of a broad range of blood group related glycolipids or glycoproteins
- 15 could be the antigen (F) of the synthetic molecule construct F-S₁-S₂-L where S₁-S₂-L is identical or equivalent to the corresponding portion of the synthetic molecule constructs designated A_{tri}-sp-Ad-DOPE (I), A_{tri}-spsp₁-Ad-DOPE (II), A_{tri}-sp-Ad-DSPE (III), B_{tri}-sp-Ad-DOPE (VI), H_{tri}-sp-Ad-DOPE (VII), H_{di}-sp-Ad-DOPE (VIII), Gal β -sp-Ad-DOPE (IX), Fu α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc-sp-Ad-DOPE (XII), and Fu α 1-2Gal β 1-3(Fu α 1-4)GlcNAc-sp-Ad-DOPE (XIII).

The structures of known blood group-related glycolipids and glycoproteins (see references) are provided in the following list:

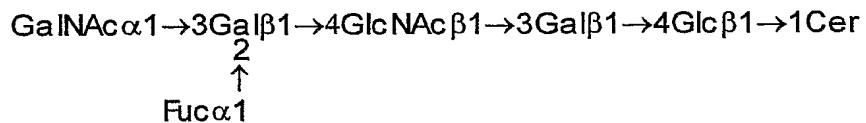
Glycolipids*

(*In general, for almost all examples of A-antigens the terminal A sugar GalNAc can be replaced with the B sugar Gal. Additionally, the lack of either the A or B determinant creates the equivalent H determinant.)

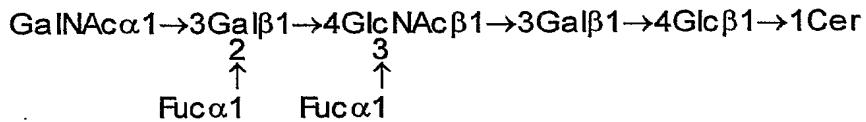
A-6-1



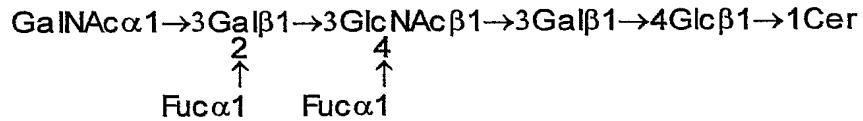
A-6-2



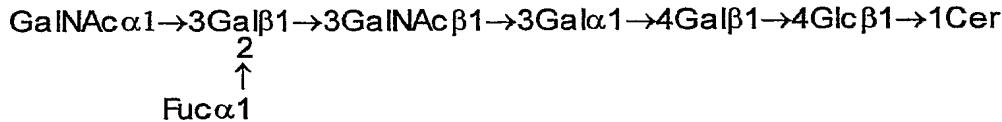
A-7-2 (ALe^y)



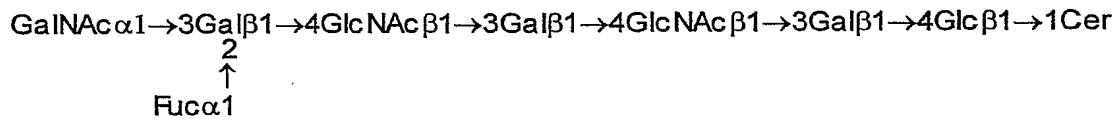
A-7-1 (ALe^b)



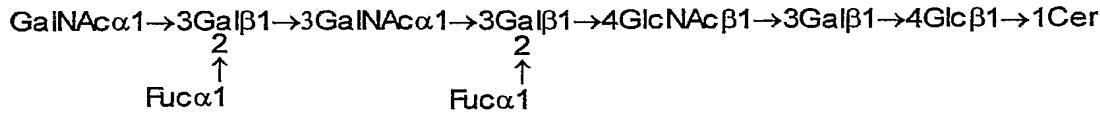
A-7-4



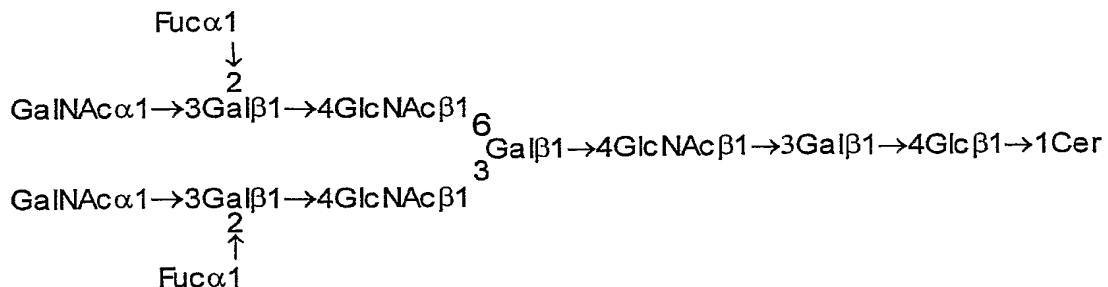
A-8-2



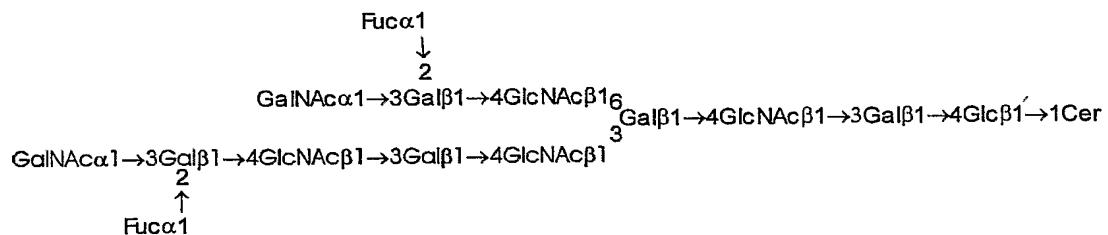
A-9-3



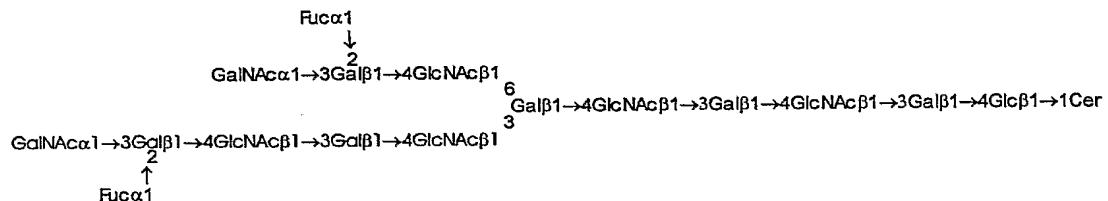
A-12-2



A-14-2



A-16-2



Lactosylceramide

$\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$

Hematoside/G_{M3}

NeuAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1Cer

Lactotriaosylceramide

GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1Cer

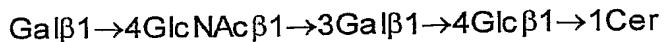
Globotriaosylceramide/P^K

$\text{Gal}\alpha 1 \rightarrow 4 \text{Gal}\beta 1 \rightarrow 4 \text{Glc}\beta 1 \rightarrow 1 \text{Cer}$

Globoside/P

$\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$

Paragloboside/neolactotetraosylceramide



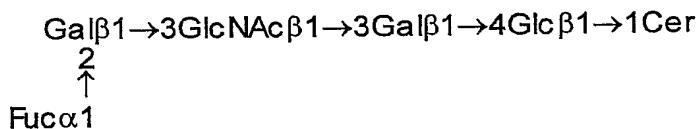
Le^c-4/Lactotetraosylceramide



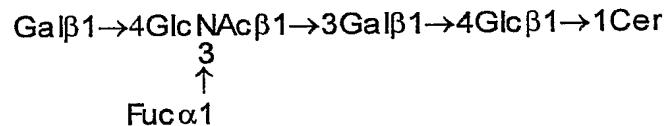
Sialoyl paragloboside/sialoyl neolactotetraosylceramide



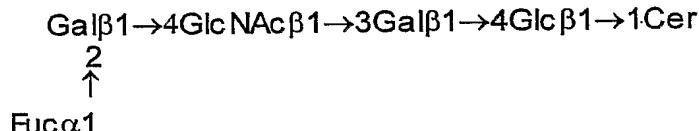
H-5-1



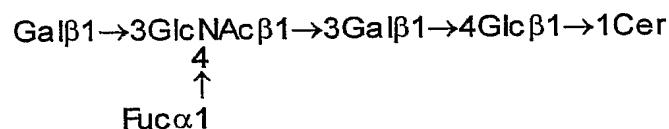
Le^x-5



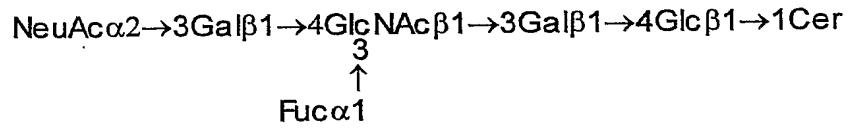
H-5-2



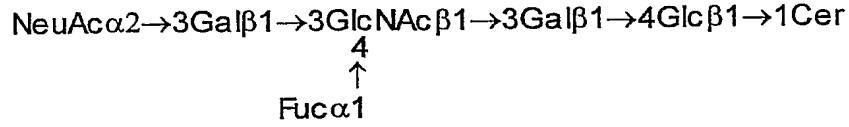
Le^a-5

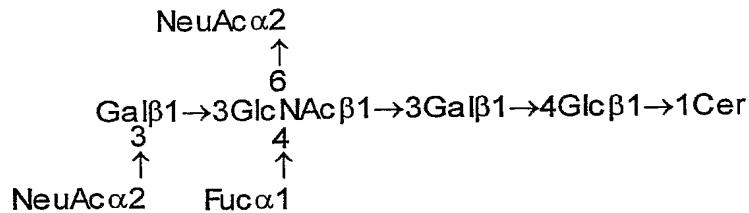
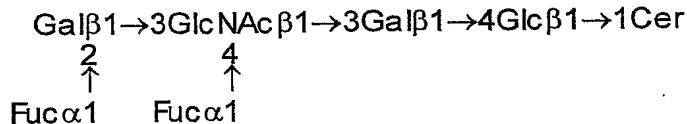
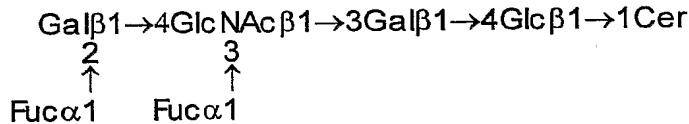


Sialyl Le^x



Sialyl Le^a-6/gastrointestinal cancer antigen (GICA or Ca 19-9)



Disialoyl Le^a-7Le^b-6Le^y-6

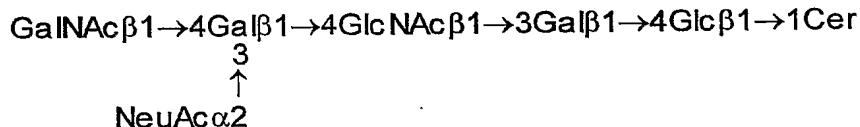
P-like



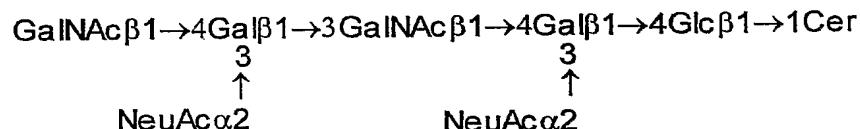
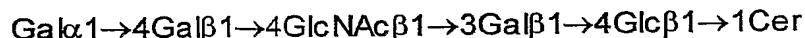
Forssman antigen



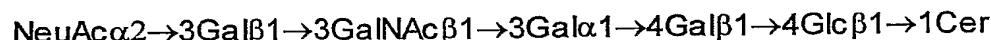
Cad erythrocyte



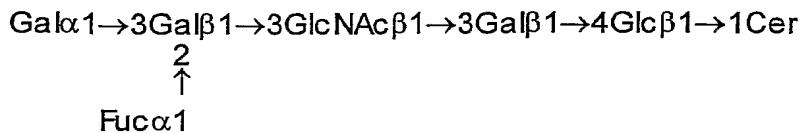
Cad hepato-carcinoma antigen

P₁

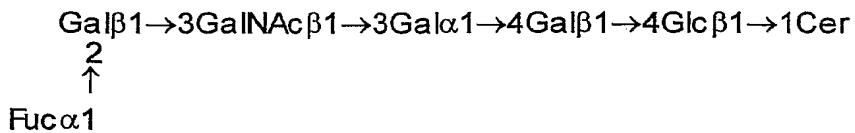
LKE/GL 7/SSEA-4



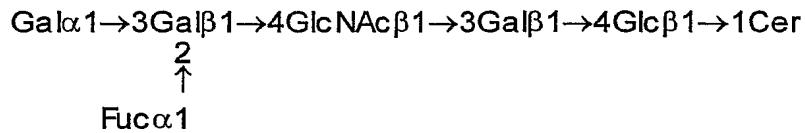
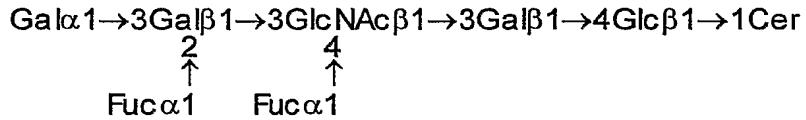
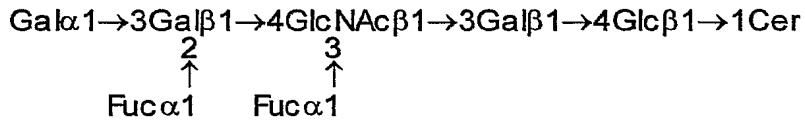
B-6-1



H-6-4



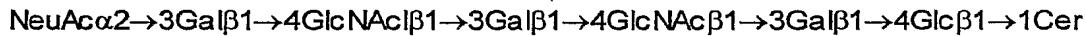
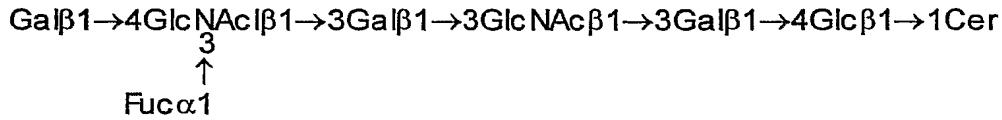
B-6-2

BLe^b-7BLe^y-7

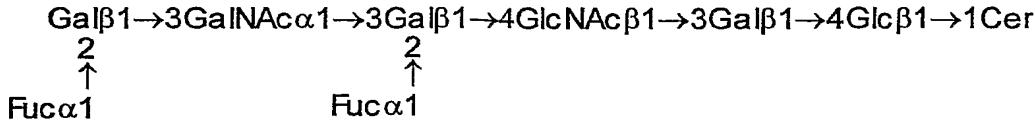
i antigen/lacto-N-nor-hexaosylceramide



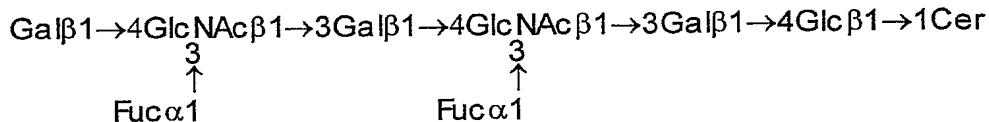
Sialyl-nor-hexaoosylceramide/sialoyl-lacto-N-nor-hexaoosylceramide

Le^x-7

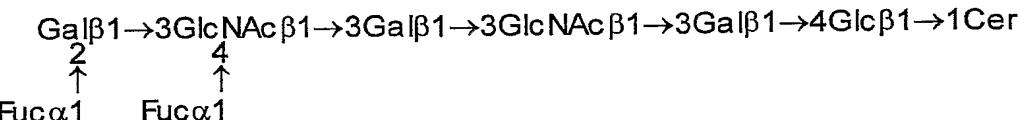
H-8-3



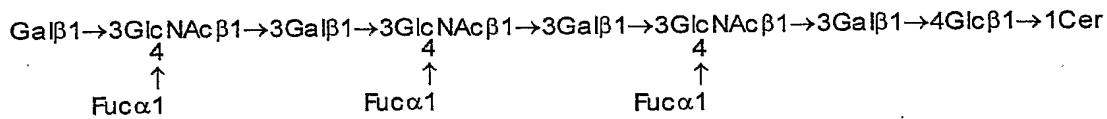
Le^x-8



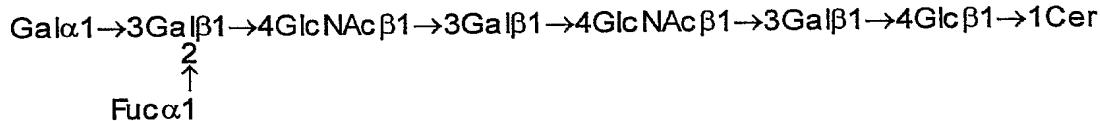
Le^b-8



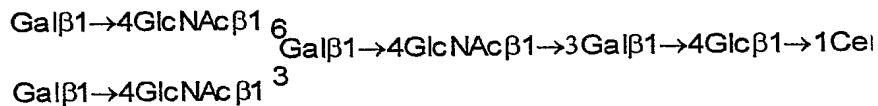
Le^a-11



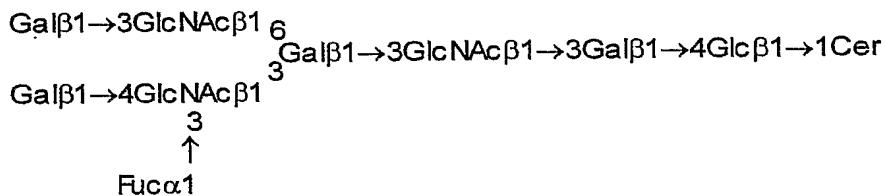
B-8-2



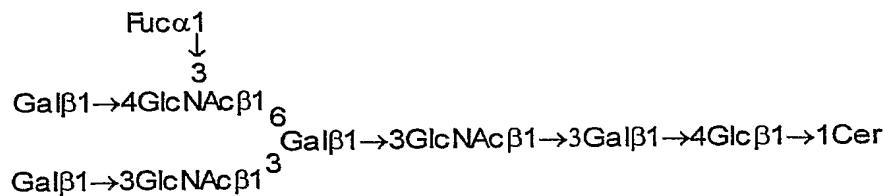
I antigen



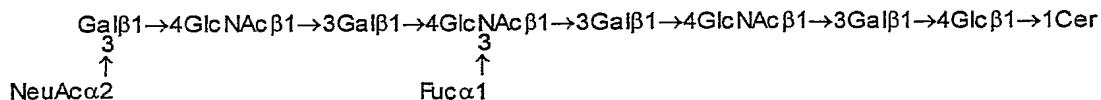
Le^c-9 (fucosylated backbone)



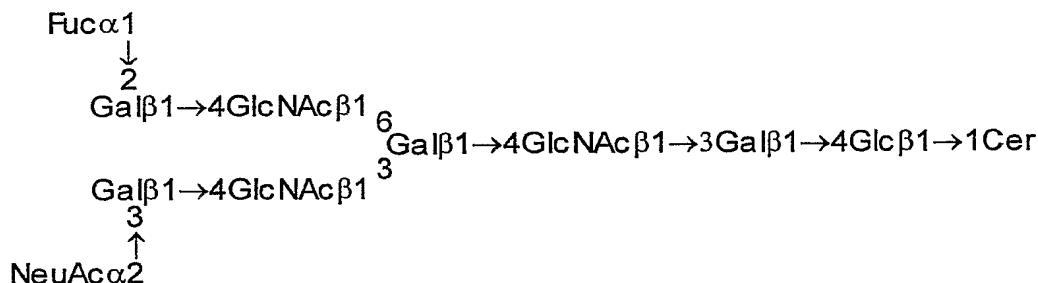
Le^c-9 (fucosylated branch)



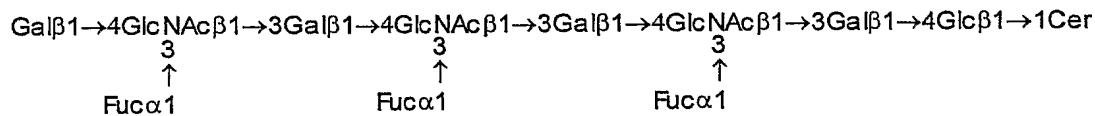
VIM-2



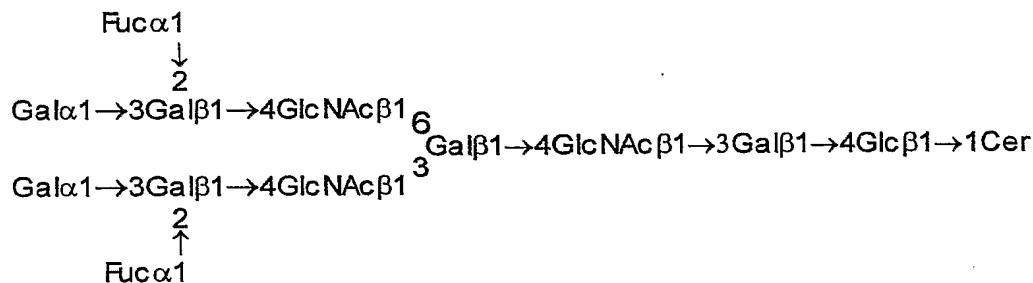
Erythrocyte F1 antigen



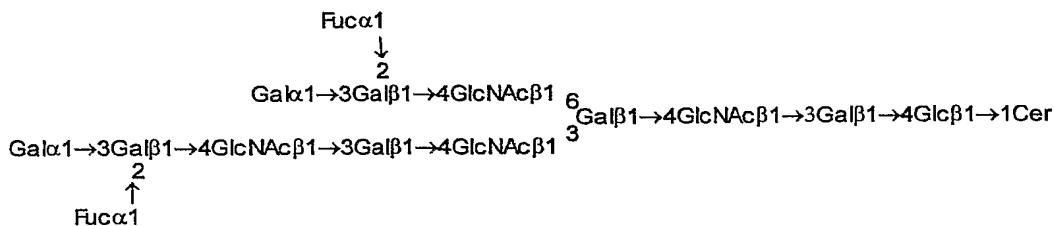
Le^x-11



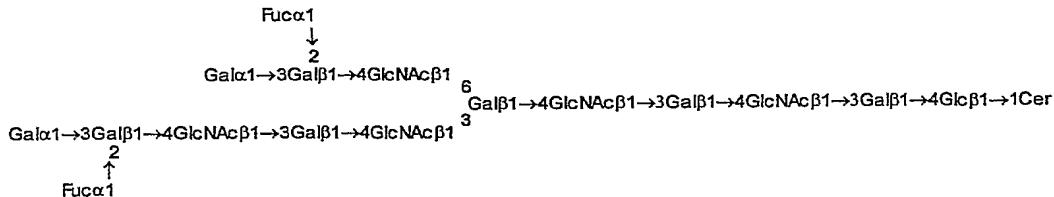
B-12-2



B-14-2



B-16-2

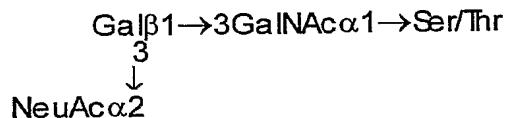


I-active polyglycosylceramide

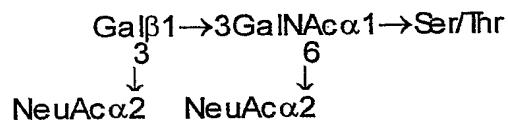


O-linked Glycoproteins

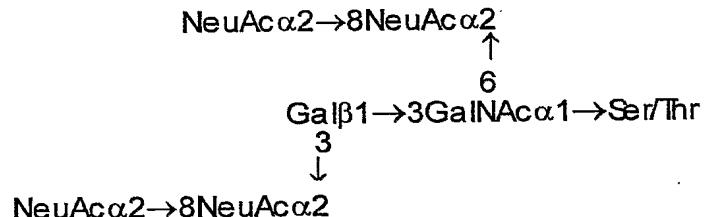
Monosialotrisaccharide



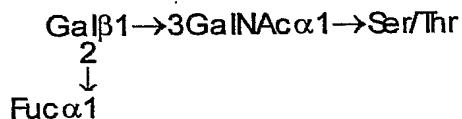
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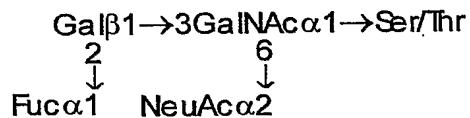
Disialoyl group oligosaccharide



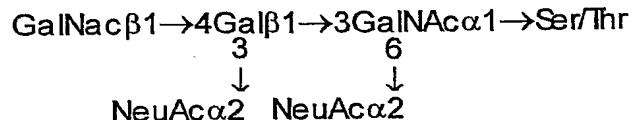
H-active trisaccharide



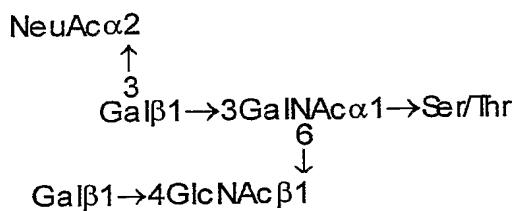
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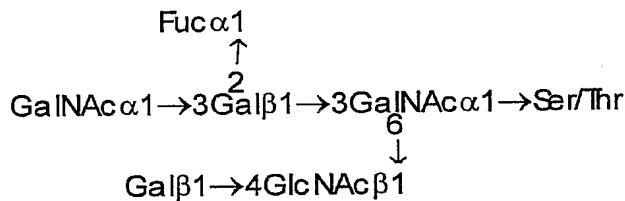
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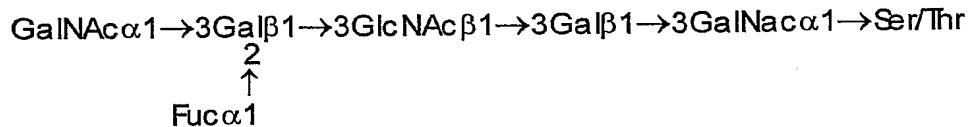
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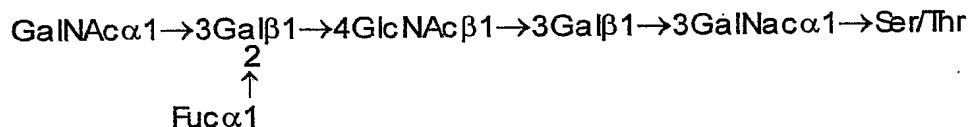
Mucin oligosaccharide/A-active glycoprotein



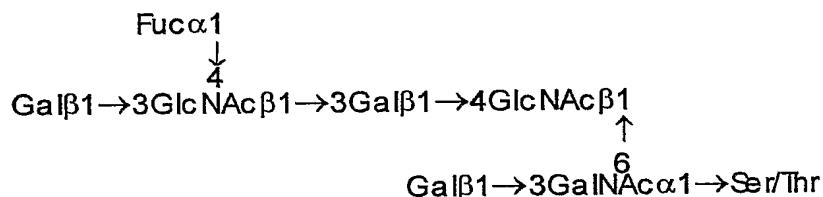
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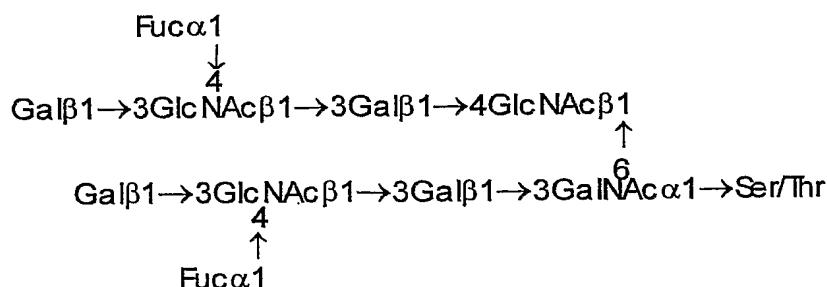
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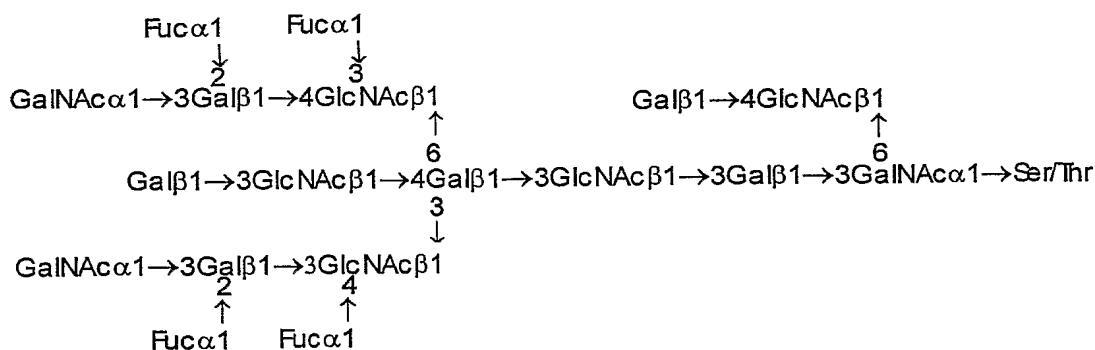
Ovarian cyst Le^a-active glycoprotein-7



Ovarian cyst Le^a-active glycoprotein-10

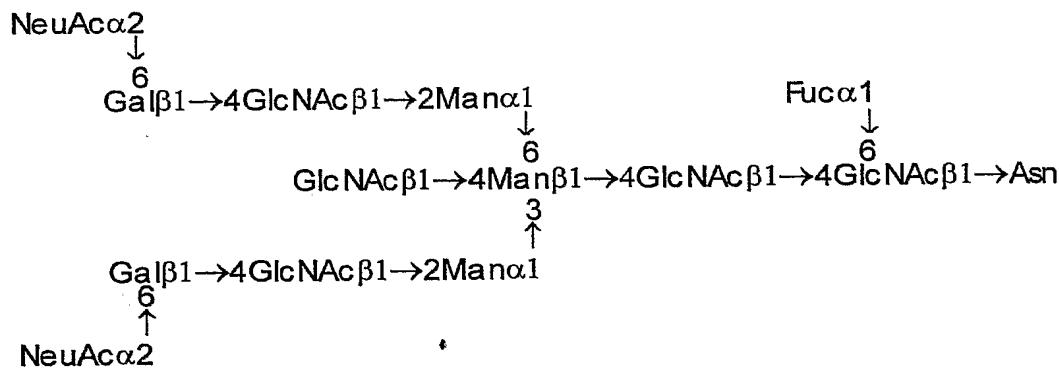


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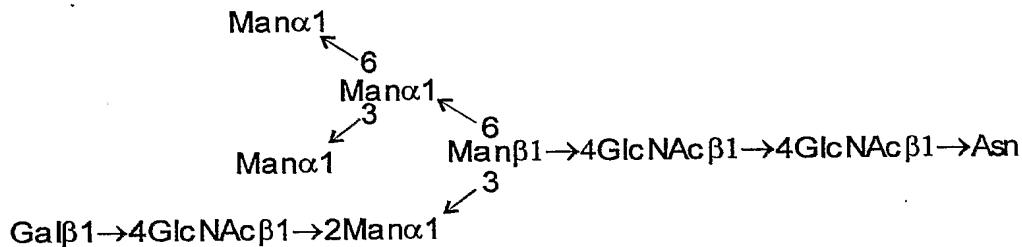


N-linked Glycoproteins

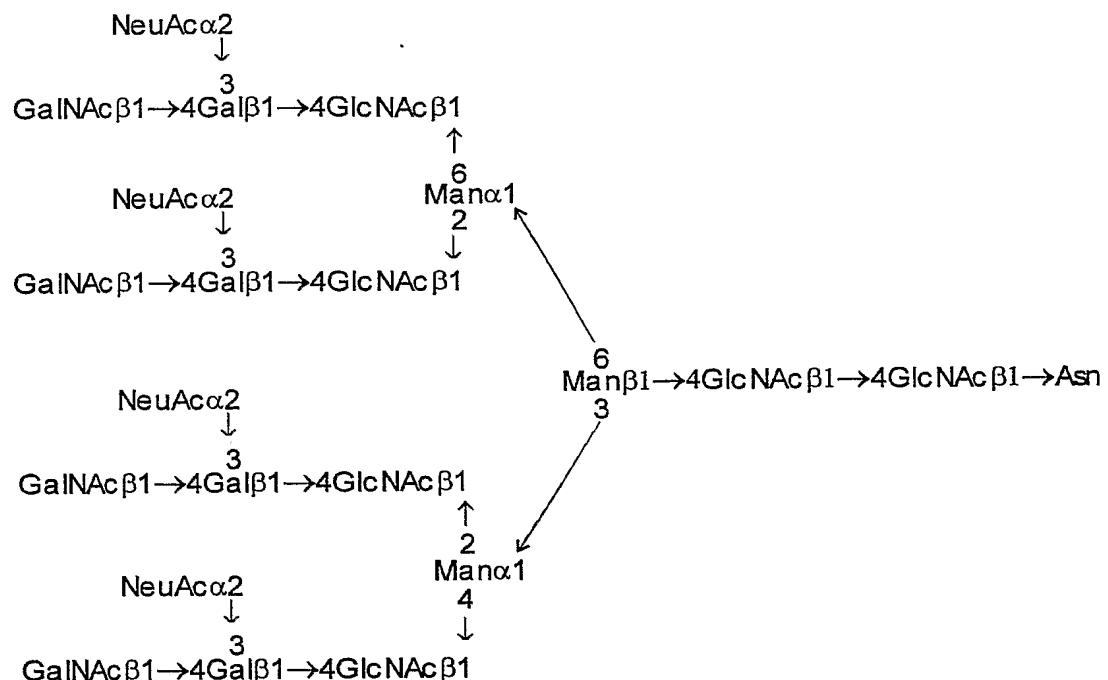
Complex type/Alkali-stable chain



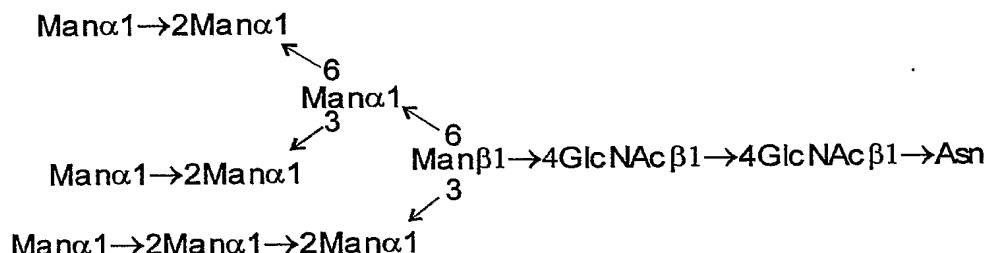
Hybrid type



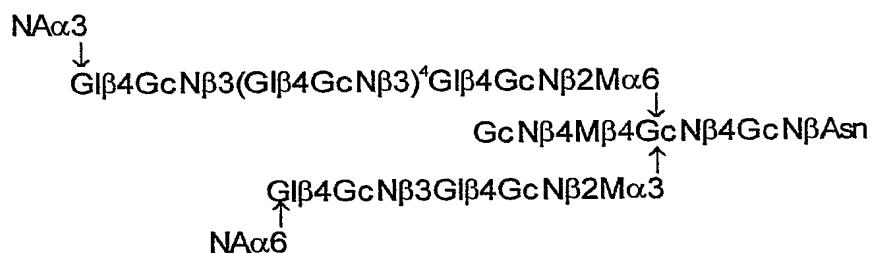
Tamm-Horsfall glycoprotein



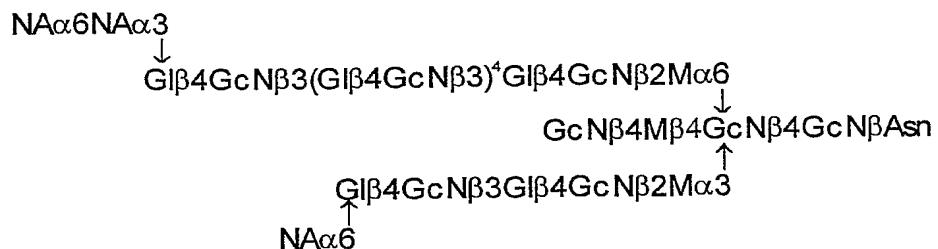
High-mannose type



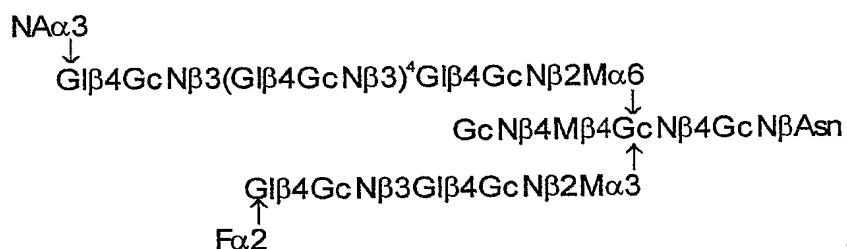
Disialyl foetal erythrocyte antigen



Trisialoyl foetal erythrocyte antigen (disialoyl group on branch)



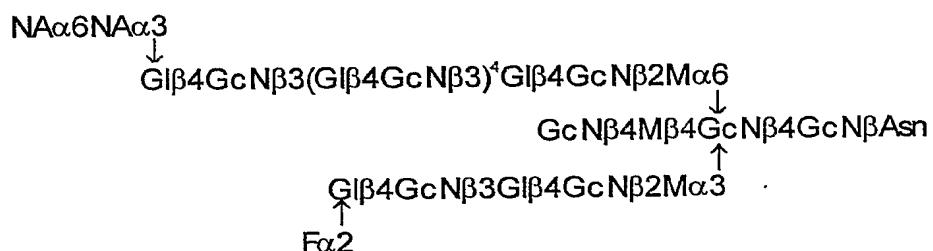
Monofucosyl-monosialyl foetal erythrocyte antigen (fucosylated backbone)



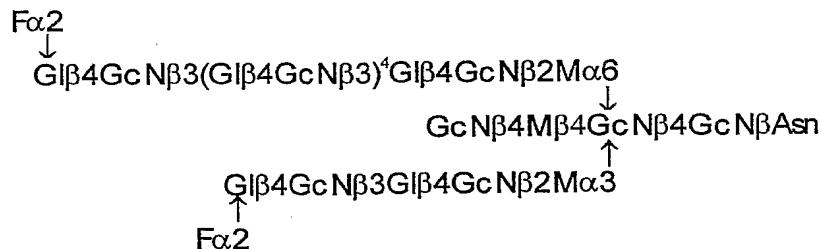
Monofucosyl-monosialyl foetal erythrocyte antigen (fucosylated branch)



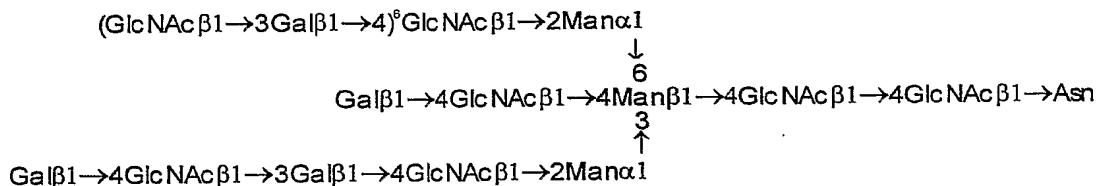
Monofucosyl-disialyl foetal erythrocyte antigen (disialyl group on branch)



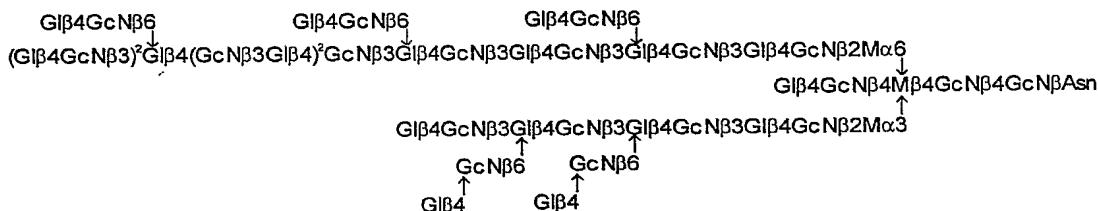
Difucosyl foetal erythrocyte antigen



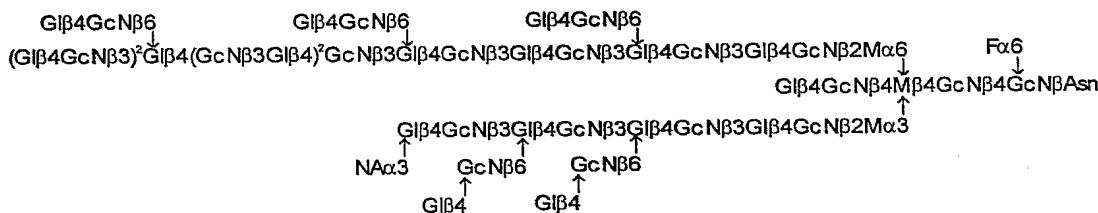
Foetal lactosaminoglycan



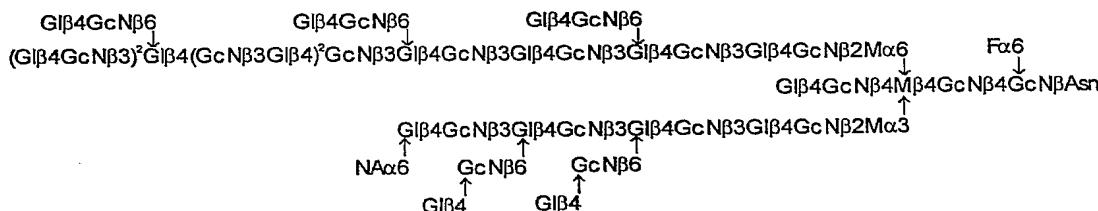
Adult lactosaminoglycan



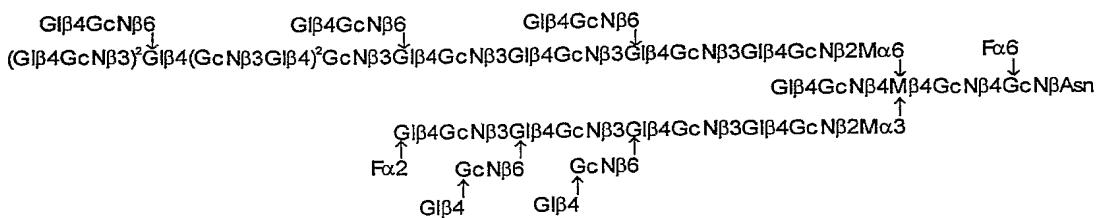
Monofucosyl-monosialoyl adult erythrocyte antigen



Monofucosyl-monosialoyl adult erythrocyte antigen



Difucosyl adult erythrocyte antigen



Key: GI = D-Gal, Gc = D-Glc, GcN = D-GlcNAc, M = D-Man, F = L-Fuc, NA = NeuAc.

It will be understood by those skilled in the art that the synthetic molecule constructs (F-S₁-S₂-L) of the invention where F is an oligosaccharide may be used as "synthetic glycolipids" and substituted for glycolipids obtained from biological (botanical or zoological) sources.

In the context of this description of the invention the term "glycolipid" means a lipid containing carbohydrate of amphipathic character including: glycosylated glycerolipids, such as glycosylated phosphoglycerides and glycosylglycerides; glycosylated sphingolipids (neutral glycolipids) such as glycosylceramides or cerebrosides; and gangliosides (acidic glycolipids).

In the context of this description of the invention the phrase "glycolipid-linked antigen" means a lipid containing carbohydrate in which an antigen (e.g. a protein) is linked to the glycolipid via the carbohydrate portion of the molecule. Examples of glycolipid-linked antigens include GPI-linked proteins.

It will be understood by those skilled in the art that a glycolipid is itself an antigen. The term and phrase "glycolipid" and "glycolipid-linked antigen" are used to distinguish between naturally occurring molecules where the antigen is the glycolipid and naturally occurring molecules where the antigen is linked to the glycolipid via the carbohydrate portion of the glycolipid. By analogy the synthetic molecule constructs of the invention could be described as both "synthetic glycolipids" and synthetic membrane anchors to the extent that the antigen may be the synthetic glycolipid *per se* or attached to the synthetic glycolipid.

It will be understood by those skilled in the art that the carbohydrate portion of a glycolipid may be modified and linked to other antigens by the methods described in the specification accompanying the international application no. PCT/NZ2003/00059 (published as WO03087346).

In the context of this description of the invention the term "glycotope" is used to refer to the antigenic determinant located on the carbohydrate portion of a glycolipid. The classification of glycolipid antigens in blood group serology is based on the structure of the carbohydrate portion of the glycolipid.

In blood group serology it is known that the terminal sugars of the glycotopes of A-antigens are GalNAc α 1-3(Fuca1-2)Gal β , and the terminal sugars of the glycotopes of the B-antigens are Gal α 1-3(Fuca1-2)Gal β . Incorporation into the membrane of RBCs of water soluble synthetic molecule constructs of the invention where F is GalNAc α 1-3(Fuca1-2)Gal β or Gal α 1-3(Fuca1-2)Gal β provides RBCs that are serologically equivalent to A-antigen or B-antigen expressing RBCs, respectively.

The terminal three sugars of the carbohydrate portion of the naturally occurring A- or B-antigen are the determinant of the A and B blood groupings. The terminal four or five sugars of the carbohydrate portion of the naturally occurring A-antigen are the determinant of the A blood sub-groupings A type 1, A type 2, etc. Accordingly the RBCs incorporating the synthetic molecule constructs of the invention can be used to characterise and discriminate between blood typing reagents (antibodies) of differing specificity.

15 Water soluble synthetic molecule constructs of the invention that exclude a carbohydrate portion are contemplated by the inventors. Antigens other than carbohydrates or oligosaccharides, but with similar physico-chemical properties, may be substituted for F in the "synthetic glycolipids" described.

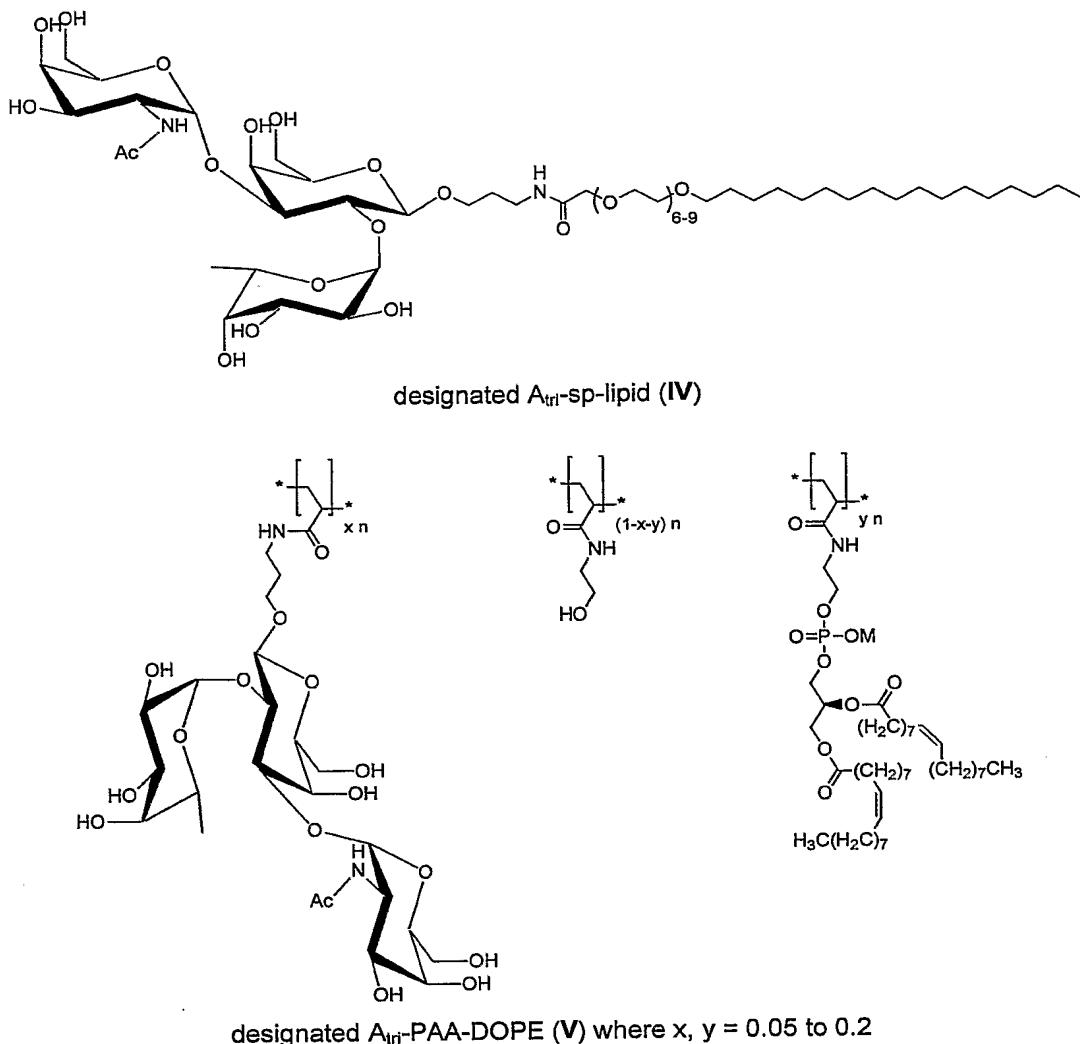
20 Synthetic molecule constructs of the invention that comprise an antigen (F) with differing physico-chemical properties to those of carbohydrates or oligosaccharides are also contemplated by the inventors. Water soluble synthetic molecule constructs comprising these antigens may be prepared by selecting different spacers.

25 The advantages provided by the synthetic molecule constructs of this invention will accrue when used in the practice of the inventions described in the specifications for the international application nos. PCT/N02/00212 (published as WO03/034074) and PCT/NZ03/00059 (published as WO03087346). The specifications accompanying these applications are incorporated herein by reference.

30 The synthetic molecule constructs overcome many of the limitations of using natural glycolipids in the practice of these inventions. A particular advantage of the synthetic molecule constructs is their superior performance and ability to be used in the transformation of cells at reduced temperatures, e.g. 4°C.

35 As described herein not all structures of the spacer (S₁-S₂) will provide a synthetic molecule construct (F-S₁-S₂-L) that is water soluble and spontaneously and stably incorporate in to a lipid bilayer such as a cell membrane. The synthetic molecule constructs designated A_{tri}-sp-lipid (IV) and Atri-PAA-DOPE (V) were determined not to be water soluble and/or unable to spontaneously and stably incorporate in to a lipid bilayer such as a cell membrane.

40



5

The invention will now be illustrated by reference to the following non-limiting Examples and Figures of the accompanying drawings in which:

10

Figure 1 shows Diamed results of Cellstab™ stored cells transformed by natural A glycolipid transformation solution at (L to R) 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL* and 1 mg/mL. Antisera used are Albaclone (top) and Bioclone (bottom). (* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and 15 washed out the next day (day 2).)

15

Figure 2 shows Diamed results of Cellstab™ stored cells transformed by natural B glycolipid transformation solution at (L to R) 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL* and 1 mg/mL. Antisera used are Albaclone (top) and Bioclone (bottom). (* - transformation solution 20 (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day (day 2)).

Figure 3 shows FACS analysis following *in vitro* transformation of human Le(a-b-) red cells with natural Le^b-6 glycolipid over time at three transformation temperatures, 37°C (top), 22°C (middle) and 4°C (bottom).

5

Figure 4 shows Diamed results of cells transformed at 4°C by A_{tri}-sp-Ad-DOPE (I) transformation solution at (L to R): washed 0.08 mg/mL; unwashed 0.08 mg/mL; washed 0.05 mg/mL; unwashed 0.05 mg/mL; washed 0.03 mg/mL; and unwashed 0.03 mg/mL. The antisera used was Bioclone anti-A.

10

Figure 5 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by A_{tri}-sp-Ad-DOPE (I) transformation solution at (L to R): 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL. The antisera used was Bioclone anti-A.

15

Figure 6 shows in the left column Diamed results of cells transformed at 4°C by B_{tri}-sp-Ad-DOPE (VI) transformation solution at (L to R): washed 0.6 mg/mL; unwashed 0.6 mg/mL; washed 0.3 mg/mL; unwashed 0.3 mg/mL; washed 0.15 mg/mL; and unwashed 0.15 mg/mL; and in the right column Diamed results of cells transformed at 4°C by B_{tri}-sp-Ad-DOPE (VI) transformation solution at (L to R): washed 0.08 mg/mL; unwashed 0.08 mg/mL; washed 0.05 mg/mL; unwashed 0.05 mg/mL; washed 0.03 mg/mL; and unwashed 0.03 mg/mL. The antisera used was Bioclone anti-B.

20

Figure 7 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by B_{tri}-sp-Ad-DOPE (VI) transformation solution at (L to R): 0.6 mg/mL, 0.3 mg/mL and 0.15 mg/mL..

25

Figure 8 shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.07 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.07 + B 0.3 mg/mL against anti-A and anti-B.

30

Figure 9 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.07 + B 0.3 mg/mL against anti-A and anti-B.

35

Figure 10 shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.07 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.07 + B 0.2 mg/mL against anti-A and anti-B.

40

Figure 11 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.07 + B 0.2 mg/mL against anti-A and anti-B.

5 **Figure 12** shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.06 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.06 + B 0.3 mg/mL against anti-A and anti-B.

10 **Figure 13** shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.06 + B 0.3 mg/mL against anti-A and anti-B.

15 **Figure 14** shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.06 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.06 + B 0.2 mg/mL against anti-A and anti-B.

20 **Figure 15** shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.06 + B 0.2 mg/mL against anti-A and anti-B.

25 **Figure 16** shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.05 + B 0.3 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.05 + B 0.3 mg/mL against anti-A and anti-B.

30 **Figure 17** shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.05 + B 0.3 mg/mL against anti-A and anti-B.

35 **Figure 18** shows Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain washed A 0.05 + B 0.2 mg/mL against anti-A and anti-B. Wells 3 and 4 contain unwashed A 0.05 + B 0.2 mg/mL against anti-A and anti-B.

Figure 19 shows cells that were no longer washed prior to testing. Diamed results of cells transformed at 4°C by parallel transformation with A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI). Wells 1 and 2 (L to R) contain unwashed A 0.05 + B 0.2 mg/mL against anti-A and anti-B.

COMPARATIVE EXAMPLES

The Comparative Examples do not form part of the invention claimed. The Comparative Examples describe red blood cell transformation with natural glycolipids.

5

Comparative Example 1 – Preparation of Natural Glycolipids*Purification by HPLC*

In the first stage, columns were packed with dry silica (15-25 µm) before each run. Relatively 10 dirty samples could be used in HPLC because the silica could be discarded along with the theoretically high level of irreversibly bound contaminants.

Glycolipids were separated on silica gel with a mobile phase of increasing polarity. The program was a linear gradient beginning with 100% chloroform-methanol-water 80:20:1 (v/v) 15 and ending with 100% chloroform-methanol-water 40:40:12 (v/v).

The HPLC equipment used was a Shimadzu system capable of pumping and mixing four separate solvents at programmed ratios. As chloroform, methanol and water evaporate at different rates, a program was developed whereby the solvent components were not mixed 20 prior to entering the HPLC.

The Shimadzu HPLC mixes four different liquids by taking a "shot" from each of four bottles in turn. "Shots" of chloroform and water directly next to each other in the lines may cause miscibility problems. Methanol was sandwiched in between these two immiscible components. 25 Additionally, the water was pre-mixed with methanol in a 1:1 ratio to further prevent problems with miscibility.

Comparative Example 2 – Transformation of Red Blood Cell Transformation with Natural Glycolipids

30

Agglutination

Transformation of red blood cells was assessed by agglutination using the Diamed-ID Micro Typing System in addition to using conventional tube serology. Diamed ABO typing cards were not used. The cards used were NaCl, Enzyme test and cold agglutinin cards, which were 35 not pre-loaded with any antisera or other reagents. This allowed the use of specific antisera with both methodologies.

Table 1. Gel-cards.

Manufacturer	Catalogue ref
Diamed	NaCl, Enzyme test and cold agglutinin cards

A comparative trial was carried out between tube serology and the Diamed system to establish the performance of the two systems. Cells were transformed at 25°C for 4 hours. Seracclone and Alba-clone anti-A sera were used to gauge equivalency. The results are shown in Table 3 below.

5

Table 2. Antisera used in comparison of tube serology with the Diamed system .

Manufacturer	Catalogue ref	Lot	Expiry
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Seracclone, Biotest	801320100	1310401	12.04.03

Table 3. Agglutination results comparing tube serology with the Diamed system.

		A glycolipid (mg/mL)				
		10	5	2	1	0
Tube	Albaclone	3+	2+	0	0	0
	Seracclone	3+	2+	0	0	0
Diamed	Albaclone	2+	2+	0	0	0
	Seracclone	3+	2+	1+	w+	0

In this experiment, the Diamed system proved to be more sensitive to the weaker reactions than tube serology with the Seracclone anti-A, but not with Albaclone. These reagents are 10 formulated differently, and are thus not expected to perform identically. However, the fact that the Seracclone anti-A tube serology combination did not detect positivity is probably due to operator interpretation. The weaker reactions are notoriously difficult to accurately score, and the difference between 1+ and 0 can be difficult to discern in tubes.

15 Optimisation

The variables of glycolipid concentration, incubation temperature, incubation duration, diluent and storage solution were examined for their effect on cell health. Efficiency and stability of transformation was assessed by agglutination with the relevant antibody.

Table 4. Tube serology agglutination of natural glycolipid A transformed cells over different times and temperatures.

A	10	5	2	1	0.1	0.01	0.001	0.0001	0
Seracclone									
(37°C for 1.5 hours)	3+	2+	0	0					0
Seracclone (25°C for 4 hours)	4+	3+	2+	1+	w+	0	0	0	0

Glycolipid Concentration

Initial transformation experiments were carried out with a highly purified (HPLC) Le^b glycolipid sample and a less pure blood group A glycolipid sample. Transformation was performed at 37°C for 1.5 hours

5

The A glycolipid sample contained other lipid impurities and thus comparatively less blood group A molecules by weight than the Le^b glycolipid sample of equivalent concentration (w/v). This seems to be borne out by the fact that higher concentrations of the A glycolipid than the Le^b glycolipid were required to produce equivalent agglutination scores (see Table 6).

10

The level of impurity in the A glycolipid sample may also have contributed to the lower stability over the 62 day period – the A-transformed cells 'died' at the highest concentration (having received the largest dose of impurity).

Table 5. Anti-A and anti-Le^b used in initial testing of natural glycolipid transformation.

Manufacturer	Catalogue ref	Batch number	Expiry
Anti-A			
Seraclone, Biotest	801320100	1310401	12.04.03
Anti-Le ^b			
CSL		12801	

15

Table 6. Stability of RBCs transformed with natural A and Le^b glycolipid as assessed by tube serology agglutination over the period of 62 days.

Glycolipid (mg/mL)	Le ^b			A		
	Day 1	Day 25	Day 62	Day 1	Day 25	Day 62
10	4+		2-3+	3+	2+	?
5	4+		2-3+	2+	2+	w+
2	3+		1-2+	0	1+	0
1	4+		2+	0	1+	0
0.1	3+	2+	0	0		
0.01	2+	2+	0	0		
0.001	2+	2+	0	0		
0.0001	2+	0	0	0		
0	0	0	0	0	0	0

The above cells were also rated for haemolysis and these results are shown in Table 7 below.

Table 7. Haemolysis as assessed visually. Day 1 – in the supernatant of the first wash after transformation; Days 25 and 62 – in the cell preservative solution before the cells are resuspended after storage. Scoring scale is analogous to the 4+ to 0 agglutination scale: hhhh – severely haemolysed, hhh – very haemolysed, hh – moderately haemolysed, h – mildly haemolysed, w – faintly haemolysed and 0 – no haemolysis seen.

Glycolipid concentration (mg/mL)	Haemolysis					
	Le ^b	A				
	Day 1	Day 25	Day 62	Day 1	Day 25	Day 62
10	h	0	h	h	h	dead
5	hh	0	hhh	w	0	hh
2	w	0	hhh	w	0	hhhhh
1	w	0	hhh	h	0	hhhhh
0.1			h			hhh
0.01			hh			
0.001			h			
0.0001			h			
Control	h	0	h	h	h	

These results show that cell haemolysis can be shown to be associated with transformation with high concentrations of glycolipid. It is unclear whether the mechanism underlying this is disruption of the plasma membrane by large amounts of glycolipid being inserted, the rate of that insertion, or is possibly due to the quantity of associated impurity. However, the results for Le^b at day 62 seem to support the first explanation.

The Le^b sample was highly purified – before being dissolved, it was a powder of pure white colour, and thus it is unlikely that the haemolysis was due to the deleterious effect of impurities. It is clear to see that at 62 days, the amount of haemolysis occurring diminishes in line with the decrease in the glycolipid concentration.

15 Incubation Temperature

Experiments were carried out to investigate other possible mechanisms for the reduction of haemolysis of RBCs during the insertion step. Previous experiments had shown that haemolysis was worse at higher glycolipid concentrations than at lower concentrations, and it is thought that haemolysis may also be related to the rate of glycolipid insertion. Since temperature is believed to affect the rate of insertion, experiments were conducted comparing transformation at 37°C with transformation at room temperature (RT; 25°C).

Since the rate was expected to slow down as temperature decreased, the incubation period for the RT experiment was 4 hrs. Haemolysis was assessed visually and scored following insertion. Serology tests were also performed on the cells. The results are shown in Table 8.

Table 8. The effect of incubation temperature on haemolysis and agglutination during insertion of glycolipids into RBC membranes. Haemolysis was scored visually at each of the three washes.

Glycolipid (mg/mL)	Haemolysis						Serology	
	RT			37 °C			RT	37 °C
	wash 1	wash 2	wash 3	wash 1	wash 2	wash 3		
10	w	0	0	hh	w	0	2+	2+
1	w	0	0	hh	h	vw	1+	w+

Incubation Duration

Incubation at 37°C was carried out for 1 and 2 hours and its effect on cell health and transformation assessed by agglutination with the relevant antibody.

5

Table 9. Antisera used in the duration of incubation trial.

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Bioclone, OCD	Anti-A, experimental reagent	DEV01102	-
Albaclone, SNBTS	Anti-B	Z0110670	01.07.05
Bioclone, OCD	Anti-B, experimental reagent	DEV01103	-

Table 10. Effect of incubation time on agglutination of cells transformed with natural glycolipids.

Glycolipid	Concentration (mg/mL)	Albaclone		BioClone	
		1 hour	2 hours	1 hour	2 hours
A	10	4+	4+	4+	4+
	5	4+	4+	4+	2+
	2	4+	3+	3+	2+
	1	3+	2+	2+	2+
	0.5	2+	2+	1+	w+
B	10	3+	2+	4+	1+
	5	3+	2+	3+	2+
	2	2+	2+	2+	1+
	1	1+	w+	1+	w+
	0.5	1+	w+	w+	w+

These results indicate that increasing the duration of incubation during natural glycolipid insertion does not enhance agglutination. In fact, the agglutination scores are reduced after 10 the two hour incubation. This may be due to the destabilisation of the membrane or exchange of the glycolipids back into solution.

Diluent

Experiments were also carried out to determine if changing the glycolipid diluent solution could reduce haemolysis. Working strength PBS was compared with 2 x PBS and 2 % Bovine Serum Albumin (BSA) in working strength PBS. Cells were incubated at 37°C for 1.5 hours.

5 The results are shown in Table 11.

Table 11. Study on the effect on haemolysis of changing the glycolipid diluent solutions during insertion of glycolipids into RBC membranes.

Glycolipid concentration (mg/mL)	Glycolipid Diluent Solution		
	PBS	2 x PBS	2% BSA in PBS
40	h hh	hhh	hhh
30	h hh	hhh	hhh
20	h hh	hhh	hhh
10	h hh	hhh	hhh
0	0	0	0

Stability

Once A and B blood group glycolipids had been HPLC purified to an acceptable level, an 10 experiment to find the appropriate concentrations for stability trials was carried out.

Table 12. Early stability trial of cells transformed with natural A glycolipid.

Expt	Day	A	10	5	2	1	0.1	0.01	0.001	0.0001	0
1	7	4+	3-4+	1+	0	0	0	0	0	0	0
2	43	3+	w+	0	0	0	0	0	0	0	0
3	50	1+	0	0	0						
4	60	3+	1+	0							
5	67	w+	vw	vw							
6	74	2+	0	0							
7	81	2+	1+	0							

Table 13. Antisera used in stability trials (Table 14 and Table 15).

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS	Anti-A.	Z0010770	12.12.04
Bioclone, OCD	Anti-A, experimental reagent	DEV01102	-
Albaclone, SNBTS	Anti-B	Z0110670	01.07.05
Bioclone, OCD	Anti-B, experimental reagent	DEV01103	-

Table 14. Tube serology of O RBCs transformed with A glycolipid in order to establish appropriate concentrations for stability trials.

Anti-A	Expt	A glycolipid (mg/mL)								
		10	5	2	1	0.5	0.1	0.01	0.001	0
Alba	1	3+	2+	1+	0		0	0	0	0
	2	4+	4+	3+	2+	w+				
Bio	1	3+	2+	1+	0		0	0	0	0
	2	4+	4+	3+	2+	w+				

1 & 2 Transformation at 25°C for 4 hours

Table 15. Tube serology of O RBCS transformed with B glycolipid in order to establish appropriate concentrations for stability trials.

Anti-B	Expt	B glycolipid (mg/mL)								
		10	5	2	1	0.5	0.1	0.01	0.001	0
Alba	1	2+	1+	w+	0		0	0	0	0
	2	1+	1+	w+	0	w+				
Bio	1	3+	2+	w+	0		0	0	0	0
	2	1+	1+	w+	0	w+				

1 & 2 Transformation at 25°C for 4 hours

5

Two sets of cells were transformed with different concentrations of natural A glycolipid. Transformation was performed at 25°C. One set of cells was tested long term, and one set of cells was tested weekly for agglutination. The agglutination results from tube serology and Diamed are shown in Table 16 below. All cells were stored in Cellstab™ in bottles with flat bases. The cells showed minimal to no haemolysis at any time.

10

Table 16. Agglutination results for cells transformed with different concentrations of natural A glycolipid. Results were obtained using Albaclone anti-A.

		A glycolipid (mg/mL)					
		10	5	2	1	0.1	control
Long term testing							
Day 1	Tube	4+	3+	2+	1+	+w	0
	Diamed	3+	3+	+w	0	0	0
Day 17	Tube	3+	2+	0	0		0
	Diamed	3+	2+	1+	0		0
Weekly testing							
Day 1	Tube	3+		2+		0	0
	Diamed	3+		0		0	0
Day 8	Tube	1+		0		0	0
	Diamed	3+		0		0	0
Day 15	Tube	1+		0		0	0
	Diamed	3+		2+		0	0
Day 22	Tube	3+		0		0	0
	Diamed	3+		0		0	0
Day 29	Tube	*+w		*0		*0	*0
	Diamed	*3+		*0		*0	*0
Day 36	Tube	*		*		*	*0
	Diamed	*3+		*0		*0	*0
Day 43	Tube	*		*		*	*0
	Diamed	*		*		*	*0

* - Albaclone, while all others used Seracclone anti-A.

Storage solution

5 Comparison of the two cell storage solutions, Celpresol™ (CSL) and Cellstab™ (Diamed) was carried out to test their relative abilities to support modified RBCs.

The stability of RBCs transformed with blood group A and B antigen solutions of varying concentrations when stored in two different cell preservative solutions – Cellstab™ and

10 Alsevers™ – was trialed.

A and B antisera from two different sources were used in serology testing.

All cells were tested using the standard tube serology platform up to 42 days, at which time the
15 cell agglutination reactions had become too difficult to score manually (see Table 17 for A results and Table 18 for B results).

Diamed gel-card testing was carried out to day 56 for the Alsevers stored cells, and discontinued at day 63 due to fungal contamination (although still returning positive scores).

The Cellstab™ stored cells continued to be tested up to day 70, and were still viable at this point (see Figure 1 for A results and Figure 2 for B results).

The reagents used in the stability trial are shown in Table 13.

5

Table 17. Tube serology results of stability trial of cells transformed with varying concentrations of A glycolipid and stored in either Cellstab™ or Alsevers™

Day	Cell storage solution	Albaclone Anti-A (SNBTS)					Bioclone Anti-A (OCD – Developmental reagent)				
		Transformation Solution (mg/mL)									
		10	5	2	2*	1	10	5	2	2*	1
2	Alsevers	4+	3+	2+	1+	w+	3+	3+	1+	1+	0
	Cellstab™	4+	4+	3+	1+	1+	3+	3+	2+	1+	0
8	Alsevers	4+	4+	2+	1+	1+	2+	2+	1+	1+	0
	Cellstab™	4+	4+	3+	2+	1+	3+	3+	2+	w+	0
14	Alsevers	4+	3+	2+	2+	w+	2+	1+	w+	vw	0
	Cellstab™	4+	3+	3+	2+	w+	3+	2+	w+	vw	0
21	Alsevers	3+	2+	2+	2+	1+	2+	2+	2+	1+	0
	Cellstab™	3+	3+	2+	+	#	2+	#	#	#	0
28	Alsevers	2+	2+	1+	1+	0	2+	2+	1+	1+	0
	Cellstab™	2+‡	2+‡	‡	‡	0	1+	w+	0	0	0
36	Alsevers	3+	2+	2+	2+	1+	3+	3+	2+	1+	1+
	Cellstab™	3+‡	2+‡	‡	‡	‡	3+‡	#	#	#	#
42	Alsevers	3+	3+	1+	w+	0	2+	2+	2+	1+	1+
	Cellstab™	4+‡	4+‡	‡	‡	‡	‡	‡	‡	‡	0

* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day.

‡ - positive cell button, but cells fall off as negative (score assignment impossible).

Table 18. Tube serology results of stability trial of cells transformed with varying concentrations of B glycolipid and stored in either Cellstab™ or Alsevers™.

Day	Cell storage solution	Albaclone Anti-B (SNBTS)					Bioclone Anti-B (OCD - Developmental reagent)				
		Transformation Solution (mg/mL)									
		10	5	2	2*	1	10	5	2	2*	1
2	Alsevers	3+	3+	1+	1+	1+	2+	1+	1+	1+	0
	Cellstab™	3+	3+	2+	2+	1+	2+	2+	2+	1+	w+
8	Alsevers	1+	1+	w+	0	0	0	0	0	0	0
	Cellstab™	2+	1+		w+	0	1+	1+	w+	0	0
14	Alsevers	2+	2+	0	w+	0	0	1+	1+	2+	0
	Cellstab™	1+	w+	0	0	0	2+	2+	w+	1+	1+
21	Alsevers	#	#	#	#	#	1	1	#	#	#
	Cellstab™	#	#	#	#	#	+	+	+	#	#
28	Alsevers	2+	1+	w+	0	0	2+	1+	2+	0	0
	Cellstab™	#	#	#	0	0	#	0	#	#	0
36	Alsevers	2+	2+	2+	1+	1+	2+	2+	2+	1+	1+
	Cellstab™	#	#	#	#	#	#	#	#	#	#
42	Alsevers	2+	2+	2+	2+	w+	2+	2+	1+	w+	w+
	Cellstab™	#	#	#	#	#	#	#	#	#	#

* - transformation solution (containing glycolipids) was not washed out after the incubation, it was left in over night and washed out the next day.

5 # - positive cell button, but cells fall off as negative (score assignment impossible).

FACS analysis of glycolipid insertion

Transformation of human Le(a-b-) red cells with natural Le^b-6 glycolipid over time at three transformation temperatures (37°C, 22°C and 4°C) was performed (Figure 3). Natural Le^b-6 glycolipid was dissolved in plasma and used to transform RBCs at a final concentration of 2 mg/mL and a final suspension of 10%.

Reactivity was determined by FACS analysis using a Gamma anti-Leb. (The serological detection level is around 10² molecules. The insertion of natural glycolipids at 4°C for 8 hours was not detectable by agglutination with antibodies.) Projection of the rate of insertion curve from FACS analysis did not indicate that the rate of insertion at 4°C would have reached agglutination detection levels within 24 hours.

Low incubation temperature

20 Transformation of RBCs with natural A or B glycolipid was performed at 37°C for 1 hour and 2°C for varying intervals. Cells were agglutinated with Bioclone anti-A or Bioclone anti-B. The results are provided in Tables 19 and 20.

Table 19. Diamed results of comparison of natural A glycolipid transformation at 37°C for 1 hour and 2°C for varying intervals.

Temp	Time (hours)	Nat A (mg/mL)				
		10	5	2	1	0
37°C	1	3+	3+	2-3+	2+	0
2°C	1	0	0	0	0	0
	4	0	0	0	0	0
	8	1-2+	0	0	0	0
	24	2-3+	2+	1-2+	0	0
	48	3+	2-3+	2-3+	0	0
	72	3-4+	3+	2+	0	0

Table 20. Diamed results of comparison of natural B glycolipid transformation at 37°C for 1 hour and 2°C for varying intervals.

Temp	Time (hours)	Nat B (mg/mL)				
		10	5	2	1	0
37°C	1	3+	2-3+	2+	0	0
2°C	1	0	0	0	0	0
	4	0	0	0	0	0
	8	0	0	0	0	0
	24	1+	0	0	0	0
	48	2+	1-2+	0	0	0
	72	2+	1+	0	0	0

The rate of transformation is slow for both natural A glycolipid and natural B glycolipid as demonstrated by the negative agglutination scores after 1 hour at 2°C. Considerable insertion at 37°C for this time interval has been demonstrated.

Natural A glycolipid insertion at 2°C required 48 hours to reach the same level of insertion obtainable by transformation at 37°C. After this time further insertion was not observed. Likewise, natural B glycolipid insertion at 2°C was not as rapid as transformation at 37°C. The agglutination scores did not improve upon continued incubation and thus seemed to have reached maximal insertion at this time point for these concentrations.

EXAMPLES

The Examples describe red blood cell transformation with the synthetic molecule constructs of the invention. In the context of these examples the term "synthetic glycolipids" is used to refer 5 to these constructs.

Example 1 - Preparation of Synthetic Glycolipids*Materials and methods*

10 TLC analysis was performed on silica gel 60 F₂₅₄ plates (Merck), the compounds were detected by staining with 8% of phosphoric acid in water followed by heating at over 200°C. Column chromatography was carried out on silica gel 60 (0.2-0.063mm, Merck) or Sephadex LH-20 (Amersham). ¹H NMR spectra were acquired on a Bruker DRX-500 spectrometer. Chemical shifts are given in ppm (δ) relative to CD₃OD.

15 *Synthesis of activated 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-O-distereoyl-sn-glycero-3-phosphatidylethanolamine (DSPE)(glycerophospholipids)*
To a solution of bis(N-hydroxysuccinimidyl) adipate (A) (70 mg, 205 μ mol) in dry N,N-dimethylformamide (1.5 ml) were added DOPE or DSPE (L) (40 μ mol) in chloroform (1.5 ml) 20 followed by triethylamine (7 μ l). The mixture was kept for 2 h at room temperature, then neutralized with acetic acid and partially concentrated in *vacuo*.

Column chromatography (Sephadex LH-20, 1:1 chloroform-methanol, 0.2% acetic acid) of the residue yielded the activated lipid (A-L) (37 mg, 95%) as a colorless syrup; TLC (chloroform-methanol-water, 6:3:0.5): R_f = 0.5 (DOPE-A), R_f = 0.55 (DSPE-A).

¹H NMR (CDCl₃/CD₃OD, 2:1), δ :

DOPE-A - 5.5 (m, 4H, 2×(-CH=CH-), 5.39 (m, 1H, -OCH₂-CHO-CH₂O-), 4.58 (dd, 1H, J=3.67, 30 J=11.98, -CCOOHCH-CHO-CH₂O-), 4.34 (dd, 1H, J=6.61, J=11.98, -CCOOHCH-CHO-CH₂O-), 4.26 (m, 2H, PO-CH₂-CH₂-NH₂), 4.18 (m, 2H, -CH₂-OP), 3.62 (m, 2H, PO-CH₂-CH₂-NH₂), 3.00 (s, 4H, ONSuc), 2.8 (m, 2H, -CH₂-CO (Ad), 2.50 (m, 4H, 2×(-CH₂-CO), 2.42 (m, 2H, -CH₂-CO (Ad), 2.17 (m, 8H, 2×(-CH₂-CH=CH-CH₂)), 1.93 (m, 4H, COCH₂CH₂CH₂CH₂CO), 1.78 (m, 4H, 2×(COCH₂CH₂-)), 1.43, 1.47 (2 bs, 40H, 20 CH₂), 1.04 (m, 6H, 2 CH₃).
35 DSPE-A - 5.39 (m, 1H, -OCH₂-CHO-CH₂O-), 4.53 (dd, 1H, J=3.42, J=11.98, -CCOOHCH-CHO-CH₂O-), 4.33 (dd, 1H, J=6.87, J=11.98, -CCOOHCH-CHO-CH₂O-), 4.23 (m, 2H, PO-CH₂-CH₂-NH₂), 4.15 (m, 2H, -CH₂-OP), 3.61 (m, 2H, PO-CH₂-CH₂-NH₂), 3.00 (s, 4H, ONSuc), 2.81 (m, 2H, -CH₂-CO (Ad), 2.48 (m, 4H, 2×(-CH₂-CO), 2.42 (m, 2H, -CH₂-CO (Ad), 1.93 (m,

4H, COCH₂CH₂CH₂CH₂CO), 1.78 (m, 4H, 2×(COCH₂CH₂-), 1,43, 1.47 (2 bs, 40H, 20 CH₂), 1.04 (m, 6H, 2 CH₃).

Condensing activated DOPE (or DSPE) with aminopropylglycoside.

5 To a solution of activated DOPE (or DSPE) (A-L) (33μmol) in N,N-dimethylformamide (1 ml) 30 μmol of Sug-S₁-NH₂ (F-S₁-NH₂) and 5μl of triethylamine were added. For example, the Sug may be either the aminopropyl glycoside (F-S₁-NH₂) of either GalNAca1-3(Fuca1-2)Galβ trisaccharide (A-glycotope) (F) or Galα1-3(Fuca1-2)Galβ trisaccharide (B-glycotope) (F).

10 The mixture was stirred for 2 h at room temperature. Column chromatography (Sephadex LH-20 in 1:1 chloroform-methanol followed by silica gel in ethyl acetate-isopropanol-water, 4:3:1 (v/v/v) of the mixture typically yielded 85-90% of the synthetic molecule construct, for example, A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE (VI).

15 ¹H NMR (CDCl₃/CD₃OD, 1:1), δ:

A_{tri}-sp-Ad-DOPE (I) - 5.5 (m, 4H, 2×(-CH=CH-), 5.43-5.37 (m, 2H, H-1 (GalNHAc) and -OCH₂-CHO-CH₂O-), 5.32 (d, 1H, H-1, J=3.5 H-1 Fuc), 2.50 (m, 4H, 2×(-CH₂-CO), 2.40 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.20 (m, 8H, 2×(-CH₂-CH=CH-CH₂-), 2.1 (s, 3H, NHAc), 1.92 (m, 2H, O-CH₂CH₂CH₂-NH), 1.8 (m, 8H, COCH₂CH₂CH₂CH₂CO and 2×(COCH₂CH₂-), 1,43, 1.47 (2 bs, 40H, 20 CH₂), 1.40 (d, 3H, J= 6.6, CH₃ Fuc), 1.05 (m, 6H, 2 CH₃).

20 A_{tri}-spsp₁-Ad-DOPE (II) - 5.5 (m, 4H, 2×(-CH=CH-), 5.43-5.37 [m, 2H, H-1 (GalNHAc) and -OCH₂-CHO-CH₂O-], 5.32 (d, 1H, H-1, J=3.6 H-1 Fuc), 2.50 (m, 4H, 2×(-CH₂-CO), 2.40- 2.32 (m, 6H, COCH₂CH₂CH₂CH₂CO and COCH₂- (sp₁), 2.18 [m, 8H, 2×(-CH₂-CH=CH-CH₂-)], 2.1 (s, 3H, NHAc), 1.95(m, 2H, O-CH₂CH₂CH₂-NH), 1.8 [m, 10H, COCH₂CH₂CH₂CH₂CO, 2×(COCH₂CH₂-...), -COCH₂CH₂ (CH₂)₃NH-], 1.68 (m, 2H, CO(CH₂)₃CH₂CH₂NH-), 1,43, 1.47 (2 bs, 42H, 22 CH₂), 1.37 (d, 3H, J=5.6, CH₃ Fuc), 1.05 (m, 6H, 2 CH₃).

25 A_{tri}-sp-Ad-DSPE (III) - 5.42-5.38 (m, 2H, H-1 (GalNHAc) and -OCH₂-CHO-CH₂O-), 5.31 (d, 1H, H-1, J=3.5 H-1 Fuc), 2.48 [m, 4H, 2×(-CH₂-CO)], 2.42 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.18 (s, 3H, NHAc), 1.95 (m, 2H, O-CH₂CH₂CH₂-NH), 1.8 [m, 8H, COCH₂CH₂CH₂CH₂CO and 2×(COCH₂CH₂-)], 1,43, 1.47 (2 bs, 56H, 28 CH₂), 1.38 (d, 3H, J=6.6, CH₃ Fuc), 1.05 (m, 6H, 2 CH₃).

30 B_{tri}-sp-Ad-DOPE (VI) - 5.5 (m, 4H, 2×(-CH=CH-), 5.42-5.38 [m, 2H, H-1 (Gal) and -OCH₂-CHO-CH₂O-], 5.31 (d, 1H, H-1, J=3.7, H-1 Fuc), 2.48 [m, 4H, 2×(-CH₂-CO)], 2.39 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.18 [m, 8H, 2×(-CH₂-CH=CH-CH₂-)], 1.93 (m, 2H, O-CH₂CH₂-NH), 1.8 [m, 8H, COCH₂CH₂CH₂CH₂CO and 2×(COCH₂CH₂-)], 1,43, 1.47 (2 bs, 40H, 20 CH₂), 1.36 (d, 3H, J=6.6, CH₃ Fuc), 1.05 (m, 6H, 2 CH₃).

35

40

H_{tri}-sp-Ad-DOPE (**VII**) - 5.5 [m, 4H, 2×(-CH=CH-)], 5.4 (m, 1H, -OCH₂-CHO-CH₂O-), 5.35 (d, 1H, H-1, J=3.2, H-1 Fuc), 4.65, 4.54 (2d, J=7.4, J=8.6, H-1 Gal, H-1 GlcNAc), 4.46 (dd, 1H J=3.18, J=12, -CCOOHCH-CHO-CH₂O-), 4.38-4.28 (m, 2H, H-5 Fuc, CCOOHCH-CHO-CH₂O-), 5 2.48 [m, 4H, 2×(-CH₂-CO)], 2.40 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.18 [m, 8H, 2×(-CH₂-CH=CH-CH₂-)], 2.08 (s, 3H, NHAc), 1.92 (m, 2H, O-CH₂CH₂CH₂-NH), 1.82-1.72 [m, 8H, COCH₂CH₂CH₂CO and 2×(COCH₂CH₂-)], 1.48, 1.45 (2 bs, 40H, 20 CH₂), 1.39 (d, 3H, J=6.5, CH₃ Fuc), 1.05 (m, 6H, 2 CH₃).

10 H_{di}-sp-Ad-DOPE (**VIII**) - 5.49 (m, 4H, 2×(-CH=CH-)), 5.37 (m, 1H, -OCH₂-CHO-CH₂O-), 5.24 (d, 1H, H-1, J=2.95, H-1 Fuc), 4.46 (d, J=7.34, H-1 Gal), 2.48 [m, 4H, 2×(-CH₂-CO)], 2.42-2.35 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.17 [m, 8H, 2×(-CH₂-CH=CH-CH₂-)], 1.95 (m, 2H, O-CH₂CH₂CH₂-NH), 1.81-1.74 [m, 8H, COCH₂CH₂CH₂CH₂CO and 2×(COCH₂CH₂-)], 1.45, 1.41 (2 bs, 40H, 20 CH₂), 1.39 (d, 3H, J=6.5, CH₃ Fuc), 1.03 (m, 6H, 2 CH₃).

15 Galβ-sp-Ad-DOPE (**IX**) - 5.51 [m, 4H, 2×(-CH=CH-)], 5.4 (m, 1H, -OCH₂-CHO-CH₂O-), 4.61 (dd, 1H J=3.18, J=12, -CCOOHCH-CHO-CH₂O-), 4.41 (d, J=7.8, H-1 Gal), 4.37 (dd, 1H, J=6.6, J=12, -CCOOHCH-CHO-CH₂O-), 2.50 [m, 4H, 2×(-CH₂-CO)], 2.40 (m, 4H, COCH₂CH₂CH₂CH₂CO), 2.20 [m, 8H, 2×(-CH₂-CH=CH-CH₂-)], 1.97 (m, 2H, O-CH₂CH₂CH₂-NH), 1.82-1.72 [m, 8H, COCH₂CH₂CH₂CH₂CO and 2×(COCH₂CH₂-)], 1.48, 1.45 (2 bs, 40H, 20 CH₂), 1.05 (m, 6H, 2 CH₃).

Example 2 – Solubility of synthetic glycolipids

25 For use in the transformation of cells the first criterion that synthetic glycolipids must satisfy is that they are soluble in aqueous solvents, e.g. phosphate buffered saline. A number of techniques, including heat and/or sonication, were employed initially in order to maximise the solubility of the synthetic glycolipids tested (Table 21).

30 The synthetic glycolipid must also be able to insert into the membrane and be recognisable to the appropriate antibody for transformation to be detected by agglutination. Initial tests on the molecules were to establish solubility and thus eliminate those molecules that were unsuitable for use in the transformation of cells.

35 The results of these initial tests are provided in Table 22.

Table 21. The range of synthetic glycolipid molecules tested.

DOPE Lipid Tails:
B _{tri} -sp-Ad-DOPE (VI)
A _{tri} -sp-Ad-DOPE (I)
Galβ-sp-Ad-DOPE (IX)
H _{di} -sp-Ad-DOPE (VIII)
H _{tri} -sp-Ad-DOPE (VII)
A _{tri} -sp _{p1} -Ad-DOPE (II)
B _{tri} -PAA-DOPE (V)
Different Lipid Tails:
A _{tri} -sp-lipid (IV)
A _{tri} -sp-Ad-DSPE (III)

Table 22. Solubility of synthetic glycolipids in hot PBS and transformation ability.

Synthetic	Water solubility	Detectable transformation ability
A _{tri} -sp-lipid (IV)	No	No
B _{tri} -PAA-DOPE (V)	No	No
B _{tri} -sp-Ad-DOPE (VI)	Yes	Yes
A _{tri} -sp-Ad-DOPE (I)	Yes	Yes
Galβ-sp-Ad-DOPE (IX)	Yes	No
H _{di} -sp-Ad-DOPE (VIII)	Yes	No
H _{tri} -sp-Ad-DOPE (VII)	Yes	Yes
A _{tri} -sp _{p1} -Ad-DOPE (II)	Yes	Yes
A _{tri} -sp-Ad-DSPE (III)	Yes	Yes

The lack of detectable transformation for Galβ-sp-Ad-DOPE (IX) and H_{di}-sp-Ad-DOPE (VIII)
 5 was thought to be due to the inability of the antibody to recognise the glycotope of these synthetic molecules. A_{tri}-sp-lipid (IV) has a single rather than a diacyl tail and it was proposed that there was no insertion of this synthetic molecule into the membrane bilayer.

Example 3 – Low temperature transformation of RBCs by A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI) synthetic glycolipids

RBCs are healthier when stored at 4°C, and likewise are believed to be healthier when transformed at 4°C. It was not thought that a significant rate of insertion of the synthetic glycolipids would occur at 4°C due to our previous studies (see Comparative Examples) and
 15 studies by others (Schwarzmann, 2000). These studies were performed with natural glycolipids. Surprisingly these studies did not predict the behaviour of the synthetic glycolipids

of the invention.

Whilst not wishing to be bound by theory, in the studies of Schwarzmann the low rate of insertion of the natural glycolipids may be due to the physicochemical properties of the natural 5 glycolipid tail; a sphingolipid and a fatty acid.

The diacyl tail of the glycolipid may be important in determining the rate of insertion. Certain diacyl tails may retain greater fluidity at lower temperatures. Alternatively, the domain of the plasma membrane into which the diacyl tail of these glycolipids inserts may retain this greater 10 fluidity.

It is known that the sphingolipid tails of natural glycolipids congregate in rigid domains and these domains may not allow further incorporation of glycolipid at low temperatures. Synthetic glycolipids with *cis*-desaturated diacyl tails may be favoured for use.

15 Transformation of RBCs with synthetic glycolipids with different lipid tails was first evaluated (Tables 22 and 24).

Table 23. Antisera used to obtain results presented in Tables 24 to 27.

Anti-A

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS		Z0010770	12.12.04

BioClone, OCD	Experimental reagent	01102	-
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Anti-B

Manufacturer	Catalogue ref	Batch number	Expiry date
Albaclone, SNBTS		Z0110600	27.04.03

BioClone, OCD	Experimental reagent	01103	-
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Table 24. Evaluation of insertion of different lipid tails by agglutination with the relevant antisera.

Molecule	Anti-sera	Transformation solution ($\mu\text{g/mL}$)										
		1000	500	250	125	100	60	50	40	30	20	10
$A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$	Alba						w+	w+	0	0	0	
	Bio						2+	1+	w+	0	0	
	Alba			4+		3+		2-				2+
	Bio				4+*		4+*		3+*			3+
	DBA	0										
$B_{\text{tri}}\text{-sp-Ad-DOPE (VI)}$	Alba	3+										
	Bio	3+										
	Alba	2+	2+	1+	0		0					
	Bio	3+	2+	1+	0		0					
$A_{\text{tri}}\text{-sp}sp_1\text{-Ad-DOPE (II)}$	Alba							0	0	0	0	0
	Bio							0	0	0	0	0
	Alba			4+		3+		2+				2+
	Bio				4+*		3-		3+*			2+
	DBA	0										
$A_{\text{tri}}\text{-sp-lipid (IV)}$	Alba	0										
	Bio	0										
$A_{\text{tri}}\text{-sp-Ad-DSPE (III)}$	Alba						0	0	0	0	0	0
	Bio						0	0	0	0	0	0
	Alba			2-		2-3+		2+				2+
	Bio			3+		2-3+		2+				2+
	DBA	0										

* - splatter.

Transformation of RBCs with synthetic glycolipids $A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$ and $B_{\text{tri}}\text{-sp-Ad-DOPE (VI)}$ at 4°C was then evaluated (Tables 25 to 28). These transformations were directed towards the preparation of cells expressing low levels of A, B or A and B glycotypes ("weak A, B and AB cells").

For the preparation of weak A and B cells transformation solutions (20 μL , $A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$ at 0.08, 0.05 and 0.03 mg/mL, and $B_{\text{tri}}\text{-sp-Ad-DOPE (VI)}$ at 0.6, 0.3, 0.15, 0.08, 0.05 and 0.03 mg/mL) in 1 x PBS were mixed with washed, packed group O RBCs (60 μL).

For the preparation of weak AB cells transformation solutions (20 μL , $A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$ at 0.07, 0.06 and 0.05 mg/mL, and $B_{\text{tri}}\text{-sp-Ad-DOPE (VI)}$ at 0.3, and 0.2 mg/mL) in 1 x PBS were combined in block titre with washed, packed group O RBCs (60 μL). The combinations were: $A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$ at 0.07 mg/mL + $B_{\text{tri}}\text{-sp-Ad-DOPE (VI)}$ at 0.3 mg/mL; $A_{\text{tri}}\text{-sp-Ad-DOPE (I)}$ at

0.07 mg/mL + B_{tri}-sp-Ad-DOPE (**VI**) at 0.2 mg/mL; A_{tri}-sp-Ad-DOPE (**I**) at 0.06 mg/mL + B_{tri}-sp-Ad-DOPE (**VI**) at 0.3 mg/mL; A_{tri}-sp-Ad-DOPE (**I**) at 0.06 mg/mL + B_{tri}-sp-Ad-DOPE (**VI**) at 0.2 mg/mL; A_{tri}-sp-Ad-DOPE (**I**) at 0.05 mg/mL + B_{tri}-sp-Ad-DOPE (**VI**) at 0.3 mg/mL; and A_{tri}-sp-Ad-DOPE (**I**) 0.05 + B_{tri}-sp-Ad-DOPE (**VI**) 0.2 mg/mL.

5

Cells and transformation solutions were placed in a 4°C fridge. Pipette mixing was performed at intervals. Cells were removed for testing at intervals against the relevant antisera and were tested in both washed and unwashed states (i.e. washed samples had the transformation solution removed).

10

After 48 hours Celpresol™ was added to the cells so that the final cells:non-cells ratio was 3:5 (v/v). The cells continued to be tested at intervals. Testing was discontinued after 10 days because cells turned brown.

15

This discolouration could be attributed to a number of factors including: cells were already 21 days old when transformed; 48 hour transformation was in PBS not Celpresol™ so cells stressed for this time; and cells may have been mishandled in transit between the transforming and testing laboratories. This may be mitigated by transformation of the cells in Celpresol™ as opposed to PBS.

20

Table 25. Diaimed results of weak A RBCs transformed at 4°C against anti-A.

Time	A _{tri} -sp-Ad-DOPE (I) (mg/mL)					
	Washed			unwashed		
	0.08	0.05	0.03	0.08	0.05	0.03
2 hrs	0	0	0	0	0	0
4 hrs	1+	0	0	2+	0	0
6 hrs	2+	0	0	2+	0	0
8 hrs	2+	0	0	2-3+	0	0
12 hrs	2-3+	0	0	3+	1+	0
24 hrs	3-4+	1+	0	3-4+	2+	0
30.5 hr	3-4+	1+	0	3-4+	2+	0
48 hrs	4+	2+	0	4+	2+	0
72 hrs	4+	2+	0	4+	2-3+	0
96 hrs	4+	2-3+	0	4+	2-3+	0
Day 7				3-4+	2+	0
Day 10				3-4+	2+	0

Table 26. Diaimed results of weak B RBCs transformed at 4°C against anti-B.

Time	B _{tri} -sp-Ad-DOPE (VI) (mg/mL)					
	washed			unwashed		
	0.6	0.3	0.15	0.6	0.3	0.15
2 hrs	0	0	0	0	0	0
4 hrs	0	0	0	1+	0	0
6 hrs	w+	0	0	1+	0	0
8 hrs	2+	0	0	2+	w+	0
12 hrs	2+	w+	0	2-3+	2+	0
24 hrs	4+	3+	2+	4+	3+	2+
30.5 hr	4+	2-3+	0	4+	2-3+	w+
48 hrs	4+	3+	1+	4+	3+	2+
72 hrs	4+	4+	2+	4+	4+	2+
96 hrs	4+	3-4+	2-3+	4+	3-4+	2-3+
Day 7				4+	2-3+	0
Day 10				4+	2+	0

Table 27. Diaimed results of weak AB RBCs transformed at 4°C in block titre against anti-A.

Day	B _{tri} -sp-Ad-DOPE (VI) (mg/mL)	A _{tri} -sp-Ad-DOPE (I) (mg/mL)					
		washed			unwashed		
		0.07	0.06	0.05	0.07	0.06	0.05
1	0.3	2+	1-2+	w+	2-3+	2+	1+
	0.2	2+	1-2+	0	2-3+	2+	1+
5	0.3	2+	1-2+	1+	2-3+	2+	1-2+
	0.2	2+	1-2+	w+	2-3+	2+	1-2+
8	0.3				2-3+	2+	2+
	0.2				2-3+	2+	1-2+

Table 28. Diaimed results of weak AB RBCs transformed at 4°C in block titre against anti-B.

Day	B _{tri} -sp-Ad-DOPE (VI) (mg/mL)	A _{tri} -sp-Ad-DOPE (I) (mg/mL)					
		washed			unwashed		
		0.07	0.06	0.05	0.07	0.06	0.05
1	0.3	3+	3+	2+	3+	3+	2-3+
	0.2	1+	1-2+	0	2+	2+	1-2+
5	0.3	2+	2+	1+	2+	2+	2+
	0.2	0	w+	vw	1+	w+	vw
8	0.3				2+	2+	2+
	0.2				1+	1+	0

Example 4 – Insertion efficiency of transformation of RBCs by A_{tri}-sp-Ad-DOPE (I) and B_{tri}-sp-Ad-DOPE (VI) synthetic glycolipids

The post-transformation supernatant solutions (from A_{tri}-sp-Ad-DOPE (I) at 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL, and B_{tri}-sp-Ad-DOPE (VI) at 0.6 mg/mL, 20 µL) were added neat and in a 1:2 dilution to washed, packed RBCs (60 µL). The tubes were incubated in a 37°C waterbath for one hour, with mixing taking place every 15 minutes.

The transformed RBCs were washed 3x with PBS and then suspended in Cellstab™ at the appropriate concentration for serology testing.

Table 29. Tube serology

Pre-trans conc (mg/mL)	Score
A _{tri} -sp-Ad-DOPE (I) at 0.08	0
1:2 of A _{tri} -sp-Ad-DOPE (I) at 0.08	0
A _{tri} -sp-Ad-DOPE (I) at 0.05	0
1:2 of A _{tri} -sp-Ad-DOPE (I) at 0.05	0
A _{tri} -sp-Ad-DOPE (I) at 0.03	0
1:2 of A _{tri} -sp-Ad-DOPE (I) at 0.03	0
B _{tri} -sp-Ad-DOPE (VI) at 0.60	vw+
1:2 of B _{tri} -sp-Ad-DOPE (VI) at 0.60	0

The score given by the post-transformation supernatant solution (from the 0.08 mg/mL pre-transformation solution) is not even that of the 0.03 mg/mL transformation solution in the first pass (w+). These results indicate that >75% of the molecules are inserted into the RBC membrane on the first pass.

In addition, the post-transformation solutions were concentrated 20x and compared in parallel with the transformation solutions of known concentration. Only the post-transformation solutions derived from the 0.08 mg/mL A_{tri}-sp-Ad-DOPE (I) and 0.6 mg/mL B_{tri}-sp-Ad-DOPE (VI) solutions were tested.

Post-transformation solutions (20 µL) were dialysed (pore size 500Da) against de-ionised water for 2 days. The samples were left to dry in a fumehood for 10 days. At the end of this time they were transferred into a rotavapor flask and set on the rotavapor to rotate under vacuum with no heat overnight.

Samples were dried in a water bath at 40°C and washed over into smaller vessels with chloroform-methanol 2:1 leaving significant amounts of dried cellular material. The chloroform-methanol 2:1 washings were dried down, washed over again into test-tubes with chloroform-methanol 2:1 and dried down. These samples were redissolved in 1 mL of 1 x PBS and used
5 for transformation experiments. The cellular material in the bottom of the flasks was washed out with water into another set of tubes.

The post-transformation solutions (from A_{tri}-sp-Ad-DOPE (I) at 0.08 mg/mL and B_{tri}-sp-Ad-DOPE (VI) at 0.6 mg/mL, 20 µL) were added to washed, packed RBCs (60 µL). In parallel, the
10 transformation solutions (A_{tri}-sp-Ad-DOPE (I) at 0.08 mg/mL, 0.05 mg/mL and 0.03 mg/mL, and B_{tri}-sp-Ad-DOPE (VI) at 0.6 mg/mL, 20 µL) were added to washed, packed RBCs (60 µL).

The tubes were incubated in a 37°C waterbath for one hour, with mixing taking place every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab™
15 at the appropriate concentration for serology testing.

Table 30. Diamed serology

conc (mg/mL)	Score
A _{tri} -sp-Ad-DOPE (I) at 0.08	3+
A _{tri} -sp-Ad-DOPE (I) at 0.05	2+
A _{tri} -sp-Ad-DOPE (I) at 0.03	1+
From A _{tri} -sp-Ad-DOPE (I) at 0.08	0
B _{tri} -sp-Ad-DOPE (VI) at 0.60	4+
From B _{tri} -sp-Ad-DOPE (VI) at 0.60	0

These results suggest that there are not enough molecules in the post-transformation solution,
20 even when concentrated 20x, to be detected by serology.

Example 5 – Transformation of murine RBCs by H_{tri}-sp-Ad-DOPE (VII) synthetic glycolipid

25 Mouse cells were transformed at 37°C for 1 hour.

Table 31. Anti-H reagents used for results in Tables 32 and 33.

Antisera	Manufacturer	Batch
Anti-H IgM	Japanese Red Cross	HIRO-75
UEA	Lorne Laboratories	11549E D.O.E. 06.2004
Bio-UEA	EY Labs	201105-2

Table 32. Tube Serology.

Cells	H Antisera		
	IgM	UEA	
		T = 0	T = 20
Mouse RBCs (- control)	0	0	0
Mouse RBCs + 0.01 mg/mL H _{tri} -sp-Ad-DOPE (VII)	0		
Mouse RBCs + 0.05 mg/mL H _{tri} -sp-Ad-DOPE (VII)	1+		
Mouse RBCs + 0.1 mg/mL H _{tri} -sp-Ad-DOPE (VII)	3+		
Mouse RBCs + 0.25 mg/mL H _{tri} -sp-Ad-DOPE (VII)	4+		1+
Mouse RBCs + 1 mg/mL H _{tri} -sp-Ad-DOPE (VII)		2+	2+
Human O RBCs (+ control)	4+	1+	2/3+
			4+

Table 33. Diamed

Cells	Score
Mouse RBCs + 0.01 mg/mL H _{tri} -sp-Ad-DOPE (VII)	0
Mouse RBCs + 0.05 mg/mL H _{tri} -sp-Ad-DOPE (VII)	0
Mouse RBCs + 0.1 mg/mL H _{tri} -sp-Ad-DOPE (VII)	2+
Mouse RBCs + 0.25 mg/mL H _{tri} -sp-Ad-DOPE (VII)	3+

5

Example 6 – Transformation of RBCs by filtered A_{tri}-sp-Ad-DOPE (I) synthetic glycolipid

Some A_{tri}-sp-Ad-DOPE (I) had been sterile-filtered through a 0.2 µm filter. To investigate whether transformation would be the same with this product a comparative trial was done.

Table 34. Anti-A used for results presented in Table 35.

Manufacturer	Catalogue ref	Batch number	Expiry date
BioClone, OCD	Experimental reagent	01102	-

Table 35. Column agglutination of A RBCs transformed with varying concentrations of sterile-filtered vs unfiltered A_{tri}-sp-Ad-DOPE (I).

Concentration (mg/mL)	Sterile-filtered A _{tri} -sp-Ad-DOPE (I)	Unfiltered A _{tri} -sp-Ad-DOPE (I)
0.2	4+	4+
0.1	4+	3-4+
0.05	2-3+	2-3+
0.01	0	0
Control 37°C	0	
Control 25°C	0	

These results show no significant difference between the two preparations of A_{tri}-sp-Ad-DOPE (I) and suggests that filtration through a 0.2 µM filter did not remove molecules or change the composition or properties of the fluid to the point that transformation was affected.

5

Example 7 – Storage of transformed cells

10 To investigate whether storage at 4°C or 37°C changed the agglutination results of A_{tri}-sp-Ad-DOPE (I) and natural A glycolipid transformed O RBCs, identified as "Syn-A" and "Nat-A" cells respectively, were divided in two and suspended to 5% in Cellstab™.

One set of cells was stored at 4°C and the other set of cells was stored at 37°C in a waterbath. Agglutination of the stored transformed cells was assessed (Table 36).

15

Table 36.

Time (hours)	Platform	Temp (°C)	Syn-A		Nat-A	Control
			A _{tri} -sp-Ad-DOPE (I) at 0.1 mg/mL	At 1 mg/mL	At 10 mg/mL	
0	Tube		3+	0	1-2+	0
20	Column	4	4+	0	3+	0
		37	4+	0	3+	0
44	Column	4	4+		3+	0
		37	4+		3+	0

Example 8 - RBC transformation with A- and B-antigen synthetic glycolipids with different non-carbohydrate structures

20

The water soluble synthetic glycolipids designated A_{tri}-sp-Ad-DOPE (I), A_{tri}-sp₁sp₂-Ad-DOPE

(II), A_{tri}-sp-Ad-DSPE (III), and B_{tri}-sp-Ad-DOPE (VI) were prepared according to the method described in Example 1 with necessary modifications.

Washed packed group O red blood cells (RBCs) (3 parts by volume) and the synthetic

5 glycolipid solution (1 part by volume, varying concentrations) were added to an eppendorf tube. The tube was incubated in a 37°C waterbath for one hour, mixing every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab™ at the appropriate concentration for serology testing.

10 Tube serology and Diamed gel-card results for RBCs transformed with the different synthetic molecule constructs are provided in Table 38. Results for the stability of the RBCs transformed with the different synthetic glycolipids at different concentrations are provided in Tables 39 to 44.

15

Table 37. Antisera used for results presented in Tables 38 to 44.

Antisera	Manufacturer	Batch
Albaclonc anti-A	SNBTS	Z0010770 – D.O.E 12.12.04
Bioclone anti-A	Ortho Diagnostics	01102 – D.O.M 16.05.02
Albaclonc anti-B	SNBTS	Z0110670 – D.O.E 12.12.04
Bioclone anti-B	Ortho Diagnostics	01103 – D.O.M 16.05.02

Table 38. Comparison of transformation of RBCs using A-antigen synthetic glycolipids at different concentrations.

Synthetic	Conc mg/mL	A Antisera			
		Albaclonc anti-A		Bioclone anti-A	
		Tube	Diamed	Tube	Diamed
A _{tri} -sp-Ad-DOPE (I)	0.25	n.d.	4+	n.d.	4+
	0.1	n.d.	4+/3+	n.d.	4+/3+
	0.05	w+	2+	2+	2+
	0.04	w+	n.d.	1+	n.d.
	0.03	0	n.d.	w+	n.d.
	0.02	0	n.d.	0	n.d.
	0.01	0	0	0	0
A _{tri} -sp-Ad-DSPE (III)	0.25	n.d.	0	n.d.	0
	0.1	n.d.	0	n.d.	0
	0.05	0	0	0	0
	0.04	0	n.d.	0	n.d.
	0.03	0	n.d.	0	n.d.
	0.02	0	n.d.	0	n.d.
	0.01	0	0	0	0
A _{tri} -sp ₁ sp ₂ -Ad-DOPE (II)	0.25	n.d.	4+	n.d.	4+
	0.1	n.d.	4+	n.d.	4+/3+
	0.05	0	3+	0	3+
	0.04	0	n.d.	0	n.d.
	0.03	0	n.d.	0	n.d.
	0.02	0	n.d.	0	n.d.
	0.01	0	0	0	0
Incubated control	–	0	n.d.	0	n.d.
Bench control	–	0	n.d.	0	n.d.

Abbreviations: n.d. Not determined

Table 39. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albacalone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	4+	4+ ^o	4+ ^o	4+ ^o
	Cellstab™	4+	4+	3+	4+ ^o	4+ ^o	4+ ^o
10	Alsevers	3+	2+	2+	4+ ^o	4+ ^o	3+
	Cellstab™	4+ ^o	3+ ^o	2+	4+ ^o	4+ ^o	4+ ^o
17	Alsevers	4+	4+	4+	4+ ^o	4+ ^o	4+ ^o
	Cellstab™	4+	4+	4+	4+ ^o	4+ ^o	4+ ^o
24	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+ ^o	4+	4+

Abbreviations: ^o splatter**Table 40.** Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at low concentrations (0.1 mg/mL, 0.05 mg/mL and 0.025 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albacalone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		0.1	0.05	0.025	0.1	0.05	0.025
2	Alsevers	3+/2+	1+	1+/w+	2+	2+/1+	1+
	Cellstab™	3+/2+	2+	1+	3+/2+	3+/2+	2+
8	Alsevers	2+	1+	w+	3+/2+	2+	2+
	Cellstab™	2+	1+/w+	w+	3+ ^o	2+	1+
15	Alsevers	2+	1+	0	3+	2+	Vw
	Cellstab™	4+	w+	0	4+	4+	1+
22	Alsevers	2+	2+	0	3+	2+	w+
	Cellstab™	4+	4+	1+	4+	4+	1+
44	Alsevers	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Cellstab™	4+	2+	w+	4+	2+	w+

5

Abbreviations: n.d. ^o Not determined
splatter**Table 41.** Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination in DiaMed gel-cards.

Day	Cell storage solution	Albacalone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+	4+	4+
10	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+	4+	4+
17	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+	4+	4+
24	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+	4+	4+
45	Alsevers	4+	4+	4+	4+	4+	4+
	Cellstab™	4+	4+	4+	4+	4+	4+
59	Alsevers	4+	4+		4+	4+	
	Cellstab™	4+	4+	4+	4+	4+	4+
73	Alsevers						
	Cellstab™	4+	4+	4+	4+	4+	4+
88	Alsevers						
	Cellstab™	4+	4+	4+	4+	4+	4+

Where there were insufficient cells for testing, blank spaces have been left.

Table 42. Stability trial of RBCs transformed with A_{tri}-sp-Ad-DOPE (I) at low concentrations (0.1 mg/mL, 0.05 mg/mL and 0.025 mg/mL). Agglutination in DiaMed gel-cards.

Day	Cell storage solution	Albacalone anti-A			Bioclone anti-A		
		Concentration of Transformation Solution (mg/mL)					
		0.1	0.05	0.025	0.1	0.05	0.025
2	Alsevers	4+	2+	0	4+	3+	1+
	Cellstab™	4+	2+	0	4+	3+	1+
8	Alsevers	4+	3+	0	4+	4+	1+
	Cellstab™	4+	3+	0	4+	4+	1+
15	Alsevers	4+	2+	0	4+	3+/2+	1+
	Cellstab™	4+	4+	0	4+	4+	1+
22	Alsevers	4+	3+/2+	0	4+	3+	w+
	Cellstab™	4+	4+	0	4+	4+	1+
29	Alsevers	4+	2+	0	4+	3+	w+
	Cellstab™	4+	3+	0	4+	4+	2+
43	Alsevers	4+	3+	w+	4+	4+	2+
	Cellstab™	4+	4+/3+	0	4+	4+	1+
50	Alsevers	4+	3+	w+	4+	4+	2+
	Cellstab™	4+	3+	0	4+	4+	1+
57	Alsevers	4+	3+/2+		4+	4+	
	Cellstab™	4+	3+	0	4+	3+	w+
63	Alsevers						
	Cellstab™	4+/3+	2+	0	4+	3+	0
71	Alsevers						
	Cellstab™	4+/3+	2+	0	4+	3+	0
86	Alsevers						
	Cellstab™	4+/3+	2+	0	4+	3+	0

Where there were insufficient cells for testing, blank spaces have been left.

Table 43. Stability trial of RBCs transformed with B_{tri}-sp-Ad-DOPE (VI) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination by manual tube serology.

Day	Cell storage solution	Albacalone anti-B			Bioclone anti-B		
		Concentration of Transformation Solution (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	3+	3+	2+	2+	1+	1+
	Cellstab™	3+	2+	2+	2+	2+	1+
9	Alsevers	4+	4+	2+	4+	3+	2+
	Cellstab™	4+	4+	3+	4+	4+	2+
16	Alsevers	4+	4+	3+	4+	4+	2+
	Cellstab™	4+	4+	2+	4+	4+	2+
23	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	3+
30	Alsevers	3+	3+	2+	2+	2+	2+
	Cellstab™	4+	3+	2+	3+ ^o	3+ ^o	2+
37	Alsevers	3+	2+	1+	3+	2+	1+
	Cellstab™	3+	3+	2+/1+	4+ ^o	3+	1+
44	Alsevers	4+	3+	1+	3+	3+	w+
	Cellstab™	4+	4+	n.d.	4+	4+	#
51	Alsevers	3+	3+	2+	4+	3+	2+
	Cellstab™	4+	4+	n.d.	4+	4+	2+

Table 44. Stability trial of RBCs transformed with B_{tri}-sp-Ad-DOPE (VI) at high concentrations (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL). Agglutination in Diamed gel-cards.

Day	Cell storage solution	Albacalone anti-B			Bioclone anti-B		
		Concentration of Transformation Solution (mg/mL)			1	0.5	0.25
		1	0.5	0.25	1	0.5	0.25
2	Alsevers	4+	4+	2+	4+	4+	2+
	Cellstab™	4+	4+	2+	4+	4+	2+
9	Alsevers	4+	4+	2+	4+	4+	2+
	Cellstab™	4+	4+	3+	4+	4+	3+
16	Alsevers	4+	4+	2+	4+	4+	1+
	Cellstab™	4+	4+	3+	4+	4+	3+
23	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	3+
30	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	3+
37	Alsevers	4+	4+	3+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	3+
44	Alsevers	4+	4+	2+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	4+/3+
51	Alsevers	4+	4+	2+	4+	4+	3+
	Cellstab™	4+	4+	3+	4+	4+	3+
58	Alsevers	4+		1+	4+		2+
	Cellstab™	4+	4+	2+	4+	4+	2+
72	Alsevers	4+		2+	4+		3+
	Cellstab™	4+	4+	3+2+	4+	4+	3+
87	Alsevers						
	Cellstab™	4+	4+/3+	1+	4+	4+/3+	2+/1+
116	Alsevers						
	Cellstab™	4+	3+	0	4+	4+/3+	1+

Where there were insufficient cells for testing, blank spaces have been left.

5 Example 9 - Red Blood Cell Transformation with H-antigen synthetic glycolipids

The water soluble synthetic glycolipids designated H_{tri}-sp-Ad-DOPE (VII), H_{di}-sp-Ad-DOPE (VIII) and Galβ-sp-Ad-DOPE (IX) were prepared according to the method described in Example 1 with necessary modifications.

10 Washed packed mouse RBCs (3 parts by volume) and the synthetic glycolipid solutions (1 part by volume of varying concentrations) were added to an eppendorf tube. The tube was incubated in a 37°C waterbath for one hour, mixing every 15 minutes. The transformed RBCs were washed 3x with PBS and then suspended in Cellstab™ at the appropriate concentration 15 for serology testing.

Tube serology and Diamed gel-card results for RBCs transformed with the different synthetic glycolipids are presented in Table 46. The results show that three sugars (H_{tri}) are required for detection by anti-H IgM, at least by the reagent used.

20

Table 45. Antisera used for results presented in Table 46.

Antisera	Manufacturer	Batch
Anti-H IgM	Japanese Red Cross	HIRO-75
UEA	Lorne Laboratories	11549E D.O.E. 06.2004
Bio-UEA	EY Labs	201105-2

Table 46. Comparison of transformation of RBCs using H-antigen synthetic glycolipids with different glycotopes made to different concentrations.

Synthetic	Conc mg/mL	H Antisera				
		IgM		UEA		Bio-UEA
		Tube	Diamed	Tube T0	Tube T20	Tube
H_{tri-sp} -sp-Ad-DOPE (VII)	1	n.d.	n.d.	2+	n.d.	2+
	0.25	4+	3+	n.d.	n.d.	1+
	0.1	3+	2+	n.d.	n.d.	n.d.
	0.05	1+	0	n.d.	n.d.	n.d.
	0.01	0	0	n.d.	n.d.	n.d.
H_{di-sp} -sp-Ad-DOPE (VIII)	0.25	0	n.d.	n.d.	n.d.	n.d.
	0.1	0	n.d.	n.d.	n.d.	n.d.
	0.05	0	n.d.	n.d.	n.d.	n.d.
	0.01	0	n.d.	n.d.	n.d.	n.d.
$Gal\beta-sp$ -sp-Ad-DOPE (IX)	0.25	0	n.d.	n.d.	n.d.	n.d.
	0.1	0	n.d.	n.d.	n.d.	n.d.
	0.05	0	n.d.	n.d.	n.d.	n.d.
	0.01	0	n.d.	n.d.	n.d.	n.d.
Human O cells	-	4+	n.d.	1+	2/3+	4+
Incubated control	-	0	n.d.	0	0	n.d.
Bench control	-	0	n.d.	n.d.	n.d.	n.d.

Abbreviations: n.d. Not determined

5

Example 10 – Insertion of H_{di-sp} -sp-Ad-DOPE (VIII) and $Gal\beta-sp$ -sp-Ad-DOPE (IX) synthetic glycolipids into murine red blood cells

10 The water soluble synthetic glycolipids designated H_{di-sp} -sp-Ad-DOPE (VIII) and $Gal\beta-sp$ -Ad-DOPE (IX) were prepared according to the method described in Example 1 with necessary modifications.

15 Murine RBCs were washed 3x in 1x PBS. 30 μ l of packed RBCs were combined with 30 μ l of H_{di-sp} -sp-Ad-DOPE (VIII), and 30 μ l of packed RBCs were combined with 30 μ l $Gal\beta-sp$ -Ad-DOPE (IX), respectively. Both synthetic molecule constructs were at a concentration of 1.0 mg/ml. 30 μ l of 1x PBS was added to 30 μ l of packed RBCs to act as the control group. Cells were incubated for 90 minutes in a 37°C shaking water-bath. RBCs were washed 3x in 1x PBS.

20 Three groups of packed RBCs were incubated with an equal volume of lectin UEA-1 for 30 minutes at room temperature. The lectin was prepared in 1x PBS at a concentration of 0.1 mg/ml. 50 μ l of a 3% cell suspension was spun for 15 seconds in an Immunofuge at low speed. Results were read by tube serology. The results are presented in Table 48. The results show that neither anti-H IgM nor UEA-1 detects two sugars (H_{di}).

Table 47. Antisera used for results presented in Table 48.

Antisera	Manufacturer	Batch
Biostest anti-H	Biostest AG	
UEA	EY Labs	201105-2

Table 48. Murine RBCs transformed with Gal β -sp-Ad-DOPE or H $_{\text{di}}$ -sp-Ad-DOPE, assessed by agglutination.

Cell Type	Inserted Molecule	UEA-1	Mouse IgM ^H
Murine RBC	Gal β (1mg/ml)	0	n.d.
Murine RBC	H $_{\text{di}}$ (1mg/ml)	0	0
Murine RBC	Control (PBS)	0	0
Human RBC	Control(PBS)	4+	3+

Abbreviations: n.d. Not determined

Example 11 – Preparation of sensitivity controls

5

The synthetic glycolipids of the invention may be used in the preparation of "sensitivity controls" (also referred to as "quality control cells", "serology controls", or "process controls") as described in the specification accompanying international application no. PCT/NZ02/00214 (WO 03/034074). The synthetic glycolipids provide the advantage that the transformation of 10 the RBCs may be achieved at reduced temperatures.

RBC transformation solutions

Two stock solutions are used:

Solution 1: 1mg/mL A_{tri}-sp-Ad-DOPE (I) suspended in Celpresol™ solution.

15 Solution 2: 5mg/mL B_{tri}-sp-Ad-DOPE (VI) suspended in Celpresol™ solution.

Glycolipids are manufactured in a white dry powder. Glycolipids in this form (enclosed in a sealed container under a controlled temperature) are stable for an indefinite period of time.

The glycolipids are suspended in solution (e.g. Celpresol™) by weight in order to formulate the 20 transformation solutions.

Once the transformation solutions are received at CSL, they are filtered (through a MILLEX®-GV 0.22 μ filter unit) under aseptic conditions.

25 *Processing of RBCs*

RBC donations are processed using a continuous flow centrifuge washer under aseptic conditions. RBC donations are washed in buffered saline followed by Celpresol™ solution. The PCV of the RBC donations is measured on a Beckman Coulter AcT Diff analyser. The donations are then adjusted to a packed cell volume (PCV) of 50% with the addition of 30 Celpresol™.

Transformation of RBCs to provide "weak AB cells"

RBCs are washed in buffered saline and Celpresol™. The cells are suspended in Celpresol™ solution to a PCV of >50%. The PCV of red cells is measured using a Beckman Coulter AcT 35 Diff. The mass of the red cell solution is weighed.

The amount of A_{tri}-sp-Ad-DOPE (I), B_{tri}-sp-Ad-DOPE (VI) and Celpresol™ for transformation is

calculated using the following equations:

$$a = \frac{P \times F}{S}$$

5

$$b = \frac{P \times F}{S}$$

$$c = P - (1 - P) - a - b$$

10

where

a = amount of A_{tri}-sp-Ad-DOPE (I) to be added per 1 mL of red cells (mL)

b = amount of B_{tri}-sp-Ad-DOPE (VI) to be added per 1 mL of red cells (mL)

15 c = amount of Celpresol™ to be added per 1mL of red cells (mL) to dilute cells to 50% PCV

P = PCV of red cell solution

F = Final desired concentration of glycolipid

S = Concentration of stock glycolipid solution

20

To determine the amount of glycolipid and Celpresol™ to add to a bulk sample of red cells, multiply each of a, b and c by the red cell volume. Add A_{tri}-sp-Ad-DOPE (I), B_{tri}-sp-Ad-DOPE (VI) and Celpresol™ to the red cell bulk sample aseptically.

25 Incubate the sample for 3 hours at 20°C under controlled temperature conditions and constant gentle agitation. At the end of the 3 hour period, aseptically remove a sample of red cells and test the sample to confirm transformation of the RBCs. Perform blood grouping using tube, tile and column agglutination technology (CAT) techniques.

30 Incubate the red cell sample for 3 hours at 2-8°C under controlled temperature conditions and constant gentle agitation for 18 hours. At the end of the 3 hour period, aseptically remove a sample of red cells and test the sample to confirm transformation of the red cells. Perform blood grouping using tube, tile and CAT techniques.

35 Wash the transformed red cells using a continuous flow centrifuge method, under aseptic conditions using Celpresol™ solution. Measure the PCV of the washed red cells and adjust to 50% PCV by the addition of Celpresol™ solution.

Formulation and dispensing

40 Aseptically combine a volume of the transformed RBCs with a volume of simulated plasma diluent (SPD). The plasma may contain monoclonal and polyclonal antibodies. Antibodies are

selected according to the desired characteristics of the sensitivity controls. The plasma may additionally contain tartrazine and bovine serum albumin.

Blood grouping and antibody screening is performed on the bulk samples using tube, tile and
 5 CAT techniques. The transformed RBC-SPD blend is then aseptically dispensed into BD Vacutainer tubes and the tubes labelled accordingly.

Validation testing

Weak AB cells produced by the use of synthetic glycolipids (designated A_wB_w in Tables 51 to
 10 53) were used to validate a range of testing platforms in parallel with naturally occurring weak A, weak B and weak AB cells.

Table 49. Reagents and cards used in validation testing.

Method	Reagent		
Tube	Epiclone		
Tile	Epiclone		
Ref	Manufacturer and type	Batch	Expiry
CAT 1	OCD BioVue ABD/Rev	ABR528A	16.06.05
CAT 2	OCD BioVue ABD/Rev	ABR521A	06.05.06
CAT 3	OCD BioVue ABD/ABD	ACC255A	24.05.05
CAT 4	Diamed ID-MTS	50092.10.02	Apr-05
CAT 5	Diamed ID-MTS Donor typing	51051.05.04	Mar-05
CAT 6	Diamed ID-MTS Recipient typing	50053.07.02	Apr-05
CAT 7	Diamed ID-MTS Cord typing	50961.08.03	Jul-05

Table 50. Testing platform methodology for validation testing.

Tile	1 drop 3% cells, 2 drops reagent, 15 min @ RT in moist chamber.
Tube	2 drops @ RT, 10 min.
ID-MTS	As per manufacturers instructions using Dil-2.
BioVue	As per manufacturers instructions using 0.8% RCD.

Table 51. Validation results across all methods against anti-A.

Cell	Type	Testing platform								
		Tube	Tile	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
1	A _x	w+	0	2+		1+	0	0	0	0
2	A _x	w+	0	2+		2+	0	0	0	0
3	A ₁ B	4+	4+	4+	4+	4+	4+	4+	4+	4+
4	A _x	w+	0	2+		2+	0	0	0	0
5	A ₂ B	3+	3+	4+		3+	3+	1+	2+	3+
6	A _x	w+	0	2+		2+	0	0	0	0
7	A _x	1+	0	2+		2+	0	0	0	0
8	A _x	w+	0	2+		2+	0	0	0	0
9	A _x	0	0	1+		1+	0	0	0	0
10	A _x	w+	0	2+		2+	0	0	0	0
11	A ₃	4+	4+	4+		3+	3+	1+	1+	3+
12	A ₃ B	3+	3+	3+		3+	2+	w+	w+	2+
13	B ₃	0	0	0	0	0	0	0	0	0
14	B ₃	0	0	0	0	0	0	0	0	0
15	A _w B _w	2+	2+	2+	2+	2+	0	0	0	0

Table 52. Validation results across all methods against anti-B.

Cell	Type	Testing platform								
		Tube	Tile	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
1	A _x	0	0	0		0	0	0	0	0
2	A _x	0	0	0		0	0	0	0	0
3	A ₁ B	4+	4+	4+	4+	4+	4+	3+	3+	4+
4	A _x	0	0	0		0	0	0	0	0
5	A ₂ B	4+	4+	4+		4+	4+	3+	3+	4+
6	A _x	0	0	0		0	0	0	0	0
7	A _x	0	0	0		0	0	0	0	0
8	A _x	0	0	0		0	0	0	0	0
9	A _x	0	0	0		0	0	0	0	0
10	A _x	0	0	0		0	0	0	0	0
11	A ₃	0	0	0		0	0	0	0	0
12	A ₃ B	4+	4+	4+		4+	4+	4+	4+	4+
13	B ₃	2+	2+	3+	2+	2+	2+	2+	2+	2+
14	B ₃	2+	2+	2+	2+	2+	2+	1+	1+	2+
15	A _w B _w	3+	3+	1+	1+	1+	0	0	0	0

Table 53. Validation results across all methods against anti-AB.

Cell	Type	Testing platform								
		Tube	Tile	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
1	A _x	3+	2+							2+
2	A _x	4+	2+							3+
3	A ₁ B	4+	4+							4+
4	A _x	3+	2+							3+
5	A ₂ B	4+	4+							4+
6	A _x	4+	4+							3+
7	A _x	4+	4+							3+
8	A _x	3+	4+							3+
9	A _x	4+	2+							2+
10	A _x	3+	4+							3+
11	A ₃	4+	4+							4+
12	A ₃ B	4+	4+							4+
13	B ₃	2+	2+							2+
14	B ₃	2+	2+							2+
15	A _w B _w	3+	3+							3+

Example 12 - Attachment of Modified Embryos to Transformed Endometrial Cells

5

The ability to effect qualitative and quantitative differences in the cell surface antigens expressed by cell types other than RBCs was investigated. The ability to enhance the adhesion of embryos to endometrial cells was adopted as a model system.

10 The synthetic molecules may be used as synthetic membrane anchors and/or synthetic molecule constructs. Therefore, they may also be employed in the method of enhancing embryo implantation as described in international patent application no PCT/NZ2003/000059 (published as WO 03/087346) which is incorporated by reference.

15 • Endometrial Cell Transformation

Insertion of water soluble synthetic molecule construct

A single cell suspension of endometrial epithelial cells was prepared. The endometrial cells were washed 3x by resuspending in CMF HBSS and centrifuging at 2000 rpm for 3 minutes.

The washed cell preparation was resuspended in 50 μ l of M2.

Micro-centrifuge tubes each containing a 50 μ l solution of 5M/ml endometrial cells were prepared. To separate tubes of endometrial cells 50 μ l of synthetic glycolipids A_{tri}-sp-Ad-

5 DOPE (I) or B_{tri}-sp-Ad-DOPE A (VI), or 50 μ l M2 were added to the control cells. The cells were incubated for 90 minutes at 37°C on a mixer. The endometrial cells were washed 3x by resuspending in CMF HBSS media and centrifuging at 2000 rpm for 3 minutes. The washed cell preparation was resuspended in 50 μ l of M2.

10 *Test For Insertion Using Fluorescent Probe:*

50 μ l of corresponding primary murine monoclonal antibody was added to each tube. Each tube was incubated at room temperature for 10 minutes. Cells were washed 3x in M2 media.

10 μ l of mouse anti-IgG FITC was added to each tube. Tubes were incubated at room temperature in dark conditions for 10 minutes. Endometrial cells were mounted on glass

15 slides and viewed under a fluorescence microscope.

Test for Direct Agglutination:

5 μ l of each group of cells was placed onto separate microscope slides. To each 5 μ l drop of cells 5 μ l of a corresponding antibody was added. The cells were gently mixed on the slide for

20 2 minutes. Agglutination was visualised under the microscope. The results are presented in Table 55.

Table 54. Antisera used for results presented in Table 55.

Antisera	Manufacturer	
Biocline anti-A	Ortho Diagnostics	01102 D.O.M. 16.05.02
Biocline anti-B	Ortho Diagnostics	Developmental reagent

Table 55. Endometrial cells transformed with A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE A (VI), as visualised using fluorescence.

Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Agglutination reaction by microscopic visualisation
Endometrial cells	A _{tri} -sp-Ad-DOPE (I) (1mg/ml)	Anti-A Biocline	4+	4+
Endometrial cells	B _{tri} -sp-Ad-DOPE (VI) (1mg/ml)	Anti-B Biocline	1+	3+
Endometrial cells	Control (M2 media)	Anti-A Biocline	0	0

25

- **Embryo Modification**

30 *Insertion of water soluble synthetic molecule construct:*

The embryo zona pellucida was removed by treating embryos with 0.5% pronase in a 37°C oven for 6 minutes or until all zones were removed. Micro-drops were prepared by adding 5 μ l

of synthetic glycolipid A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE (VI), at a concentration of 1 mg/mL to a 45µl drop of M2 media overlaid with mineral oil. All embryo groups were incubated in the 50µl micro-drops for 1 hour at 37°C. Embryos from experimental and control groups were washed 3x with M2 media.

5

Test for Insertion:

Embryos from experimental and control groups were placed into a micro-drop of corresponding antibody and incubated for 30 min at 37°C. Embryos from experimental and control groups were washed 3x with M2 media.

10

Embryos from all experimental and control groups were placed into micro-drops of anti-mouse Ig FITC (1:50 dilution anti-mouse Ig FITC in M2) and incubated for 30 min at 37°C. Embryos from experimental and control groups were washed 3x with M2 media. Embryos were mounted on microscope slides in a 5µl drop of M2 and the drops overlaid with oil.

15

The slides were viewed under a fluorescence microscope. Results are presented in Tables 56 and 57. The negative result for transformation with B_{tri}-sp-Ad-DOPE (VI) is attributed to a lack of 1° antibody sensitivity.

Table 56. Embryos transformed with A_{tri}-sp-Ad-DOPE (I) as visualised using fluorescence.				
Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Embryo Morphology 24hr post insertion
Embryos	A _{tri} -sp-Ad-DOPE (I)	Anti-A Biocloner	2+/1+	Appeared viable
Embryos	Control	Anti-A Biocloner	0	Appeared viable

20

Table 57. Embryos transformed with A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE (VI), as visualised using fluorescence.				
Cell Type	Inserted Antigen	1° antibody	Fluorescence score after incubation with IgFITC Probe	Embryo Morphology 24hr post insertion
Embryos	A _{tri} -sp-Ad-DOPE (I)	Anti-A Biocloner	2+	n.d.
Embryos	B _{tri} -sp-Ad-DOPE (VI)	Anti-B Biocloner	0	n.d.
Embryos	Control (M2 media)	Anti-A Biocloner	0	n.d.

Abbreviations: n.d. Not determined

Enhanced Attachment Transformed Endometrial Cells to Modified Embryos

Modified embryos (BioG-Avidin-BioIgG^B and BioG-Avidin-BioIgM^A) were prepared in accordance with the methods described in the specification accompanying the international application no. PCT/NZ03/00059 (published as WO03/087346).

Two concave glass slides were prepared, one with two wells of synthetic glycolipid A_{tri}-sp-Ad-

DOPE (I) inserted endometrial cells and the other with two wells of synthetic glycolipid B_{tri}-sp-Ad-DOPE (VI) inserted endometrial cells.

The two groups of embryos were transferred to each of the concave glass slides:

5

Slide 1 A_{tri}/IgG^B embryos
A_{tri}/IgM^A embryos

10

Slide 2 B_{tri}/IgG^B embryos
B_{tri}/IgM^A embryos

The embryos were surrounded with endometrial cells. The wells were covered with mineral oil and incubated for 15 minutes at 37°C. Using a wide bore handling pipette each group of embryos were carefully transferred to a fresh drop of M2 media. The embryos were gently

15 washed. The embryos were gently transferred into 2µL of M2 media on a marked microscope slide. Each drop was overlaid with mineral oil

Under a central plane of focus on an Olympus microscope the number of endometrial cells adhered to the embryos in each group was assessed. The number of cells adhered to each 20 embryo was recorded. Results are presented in Table 58.

Table 58. Endometrial cells transformed with A_{tri}-sp-Ad-DOPE (I) or B_{tri}-sp-Ad-DOPE (VI), and embryos modified with BioG-Avidin-BiolgG^B or BioG-Avidin-BiolgM^A. Assessment by attachment of endometrial cells to embryos.

Cell Type	Transformed endometrial cells	Modified embryos	Average number of endometrial cells attached to modified embryos
Endometrial cells	A _{tri} -sp-Ad-DOPE (I)	BioG-Avidin-BiolgG ^B	2.3
		BioG-Avidin-BiolgM ^A	7.25
Endometrial cells	B _{tri} -sp-Ad-DOPE (VI)	BioG-Avidin-BiolgG ^B	6.7
		BioG-Avidin-BiolgM ^A	3.4

Where in the foregoing description reference has been made to integers or components 25 having known equivalents then such equivalents are herein incorporated as if individually set forth.

Although the invention has been described by way of example and with reference to possible embodiments thereof it is to be appreciated that improvements and/or modification may be 30 made thereto without departing from the scope or spirit of the invention.

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